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Original Article

Evaluation of recent methods versus conventional methods for diagnosis of early-onset neonatal sepsis

Ali Kh Al-Zahrani[1], Mabrouk M Ghonaim[2,3], Yousri M Hussein[2,4], Emad M Eed[2,3], Amany S Khalifa[5,6], Lalia S Dorgham[2,7]

[1] *Department Pediatrics, College of Medicine Taif University, Neonatal Intensive Care Unit King Abdulaziz Specialist Hospital, Taif, Saudi Arabia*
[2] *Medical Laboratories Department, Faculty of Applied Medical Sciences, Taif University, Saudi Arabia*
[3] *Medical Microbiology & Immunology Department, Faculty of Medicine, Menoufia University, Egypt*
[4] *Medical Biochemistry Department, Faculty of Medicine, Zagazig University, Egypt*
[5] *Medical Microbiology and Immunology Department Faculty of pharmacy, Taif University, Saudi Arabia*
[6] *Clinical pathology Department, Faculty of Medicine, Menoufia University, Egypt*
[7] *Community Medicine Department, National Liver Institute, Menoufia University, Egypt*

Abstract

Introduction: Hospital-acquired infections continue to be a major public health problem, especially among neonates. Large proportions of infants are admitted to neonatal intensive care units (NICUs) and receive potent systemic antibiotics while the diagnostic work-up is still in progress. This study aimed to evaluate the recent methods for diagnosing neonatal sepsis (NS) and compare them to conventional diagnostic work-up.

Methodology: The study included 100 neonates divided into three groups: proven early-onset NS, clinical early-onset NS, and negative infectious status. Bacterial DNA was detected in the blood by broad-range 16S rDNA polymerase chain reaction (PCR). Markers for diagnosis of bacterial infection, which includedprocalcitonin (PCT), interleukin-6 (IL-6), and highly sensitive C-reactive protein (hs-CRP), were measured by enzyme-linked immunosorbent assay (ELISA).

Results: Blood culture was positive in 25 cases, while PCR for 16S rDNA was positive in 32 cases. Hs-CRP was significantly elevated in 30 patients in group 1, 35 patients in group 2, and 8 patients in group 3. IL-6 was significantly elevated in 28 patients in group 1, 24 patients in group 2, and 9 patients in group 3. PCT was found to be significantly elevated in 29 patients in group 1, 31 patients in group 2, and 2 patients in group 3.

Conclusions: The16S rDNA PCR assay was more sensitive than blood culture. The combination of markers (hs-CRP, PCT, and IL-6) is better than single markers to diagnose sepsis. PCT had greater diagnostic value than did hs-CRP and IL-6, while IL-6 was better for diagnosis of neonatal infection.

Key words: neonatal sepsis; PCR; procalcitonin; IL-6; *hs*-CRP.

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Introduction

Neonatal sepsis (NS) has significant morbidity and mortality rates and is still difficult to diagnose on presentation. For this reason, neonates with suspected sepsis are usually prone to empiric broad-spectrum systemic antibiotic therapy until sepsis can be excluded, and pathogens are identified in only a small proportion of those patients. This empirical overuse of antibiotics favors the development of antimicrobial-resistant organisms [1,2]. The outcome of sepsis in neonates is directly related to the early management of the infection, and hence prompt use of proper antibiotic therapy is absolutely essential. In order to prevent microbial resistance induced by unnecessary administration of antibiotics, a definite diagnosis should be made using laboratory tests with higher diagnostic value [3,4].

The gold standard for diagnosis of bacterial sepsis is blood culture, although pathogens in blood cultures are only detected in approximately 25% of cases. The sensitivity of blood culture is suspected to be low; therefore, the diagnosis of sepsis is often based on clinical assessment, in combination with laboratory markers such as C-reactive protein (CRP) [2].

Procalcitonin (PCT) is produced in the liver or by macrophages and is secreted as an acute-phase reactant

into the blood during infection. PCT starts to rise four hours after exposure to bacterial products and reaches peak concentration within six to eight hours. Recently, PCT has received considerable interest in diagnosis of bacterial infections in both pediatric and adult populations, although its accuracy in NS diagnosis is still controversial [57]. In a study by Vazzalwar *et al*. [8], a PCT cutoff value of 0.5 ng/mL was found to be more sensitive than CRP in predicting late-onset NS in very low birth weight infants. In that study, PCT level was elevated during early-onset and late-onset NS, and its overall value as a diagnostic test was comparable with CRP [8]. One limitation of PCT as an early marker for early-onset NS is that its concentration continues to rise normally during the first 48 hours after birth, with a peak concentration at 18 to 30 hours [9,10].

Pro-inflammatory cytokines are primarily responsible for initiation of effective defense against invasive pathogens. Interleukins, especially interleukin-6 (IL-6), was suggested to be used for diagnosis of bacterial infection in neonates [3]. IL-6 is produced by phagocytes and endothelial cells in response to infection and inflammation. Its serum level rises rapidly in the early stages of the inflammatory response, preceding the increase in CRP, and is followed by a rapid decline. Studies have reported that IL-6 cutoff values to diagnose sepsis have ranged from 18 to 31 pg/mL [11,12]. However, elevation of cytokines may occur normally after delivery, limiting their use as a diagnostic tool in the neonatal setting, especially in the immediate postpartum period. There are also several other variables, such as hypoxia, fetal distress, prematurity, antenatal steroids, and other meconium aspiration, that elevate cytokine levels and limit their use in diagnosing early-onset NS [13].

Detection of bacterial DNA in blood samples of neonates is suggested to be a rapid and sensitive supplement to blood culture in diagnosis of neonatal bacterial sepsis. However, to our knowledge, there are no available standardized, clinically evaluated methods for the detection of bacterial DNA in blood samples from neonates [2,14].

This study aimed to evaluate the new methods for diagnosis of NS (broad-range 16S rDNA polymerase chain reaction [PCR] done on whole blood samples without prior enrichment and determination of PCT and IL-6 levels) and compare them to the conventional BACTEC blood culture. Because suspicion of NS is based on a number of known risk factors, clinical signs, and laboratory markers, identification of a sign or a marker that could predict the diagnosis of NS is of paramount importance.

Methodology

Patients

This study included 100 patients with suspected NS who were admitted to King Abdul Aziz Specialist Hospital at Taif Saudi Arabia, between January 2013 and January 2014. Before the neonates were enrolled in the study, informed consent was obtained from each neonate's father. Demographic, clinical, and laboratory data were collected on structured data collection sheets. Newborns were considered eligible if they showed clinical signs of sepsis in their first week of life. Clinical signs of NS included respiratory manifestations such as apnea (suspension of external breathing for at least 10 seconds); tachypnea (respiratory rate over 70 breaths per minute in preterm and over 60 breaths per minute in term neonates); nasal flaring; retractions; cyanosis or respiratory distress; bradycardia (heart rate less than 100 beats per minute [bpm] in preterm and less than 80 bpm in term neonates) or tachycardia (the upper threshold of heart rate based on age: 1-2 days, > 159 bpm; 3-6 days, > 166 bpm); hypotonia or seizures; poor skin color; and irritability or lethargy [15]. Neonates suspected to have congenital malformations and/or laboratory-confirmed TORCH infections were excluded from the study.

Blood sampling

Blood samples were aseptically obtained from each neonate within the 24 hours of NICU admission as follows: 0.5 mL was inoculated immediately into blood culture bottles for blood culture, 0.5 mL was collected in an ethylenediaminetetraacetic acid (EDTA) tube for PCR, and 1 mL was collected in plain tubes to separate serum, which was kept at -20°C until used for assessment of hs-CRP, IL-6, and PCT.

Laboratory methods

Blood culture bottles were directly incubated in BACTEC system (Becton Dickinson, New Jersey USA). Positive cases were subjected to subculture and complete bacteriological identification according to the standard microbiological methods [1]. IL-6 measurement was performed using a quantitative sandwich enzyme-linked immunoassay (Abcam, Cambridge, UK). Also, hs-CRP and PCT were assessed by enzyme immune assay (Abcam) [6]. Broad-range bacterial 16S rDNA was detected by PCR. DNA was extracted from blood by a DNA

purification kit (Promega, Madison, USA) according to the manufacturer's instructions. PCR reactions were set up to amplify bacterial DNA using the primer (Macrogen, South Seoul, Korea) 5'TGAAGAGTTTGATCATGGCTCAG combined with 5'TACCGCGGCTGCTGGCA and cycling conditions described previously [2]. The primers react with highly conserved regions of the bacterial 16S rRNA gene to provide PCR products of approximately 500 base pairs. The amplified DNA products were separated and visualized by electrophoresis on 1.2% agarose gels containing 0.5 µg of ethidium bromide per milliliter. The gels were seen and photographed under UV transillumination [2].

Results

The studied neonates were divided into three groups: group 1, proven early-onset NS; group 2, clinical early-onset NS; and group 3, negative infectious status or non-sepsis. Group 1 included neonates with positive blood culture and/or positive PCR results for bacterial 16S rDNA. Group 2 included neonates with negative blood culture who had positive clinical signs consistent of sepsis, and positive sepsis screen based on band cells > 20%, polymorphocytosis, elevated CRP level, and other parameters according to Malik *et al*. [15]. Group 3 included neonates suspected of having sepsis who had negative blood culture and negative sepsis screen (Table 1).

Blood cultures were positive in 25 cases; 2 of them were found to be negative for 16S rDNA. The most common isolated organisms were Gram-negative bacilli (*Klebsiella*, *E.coli*, *Enterobacter*, *Serratiamarcescens*, and *Acinetobacter*), while Gram-positive organisms (*Streptococcus agalactiae* [GBS], coagulase-negative staphylococci [CoNS], *Staphylococcusaureus*,and *Enterococcus*) were less common, as shown in table 3. PCR for 16S rDNA was positive in 32 cases; 7 of them showed negative blood culture (Table 2). The hs-CRP was significantly elevated (cut-off value = 2.5mg/L [2]) in 30 patients in group 1, 35 patients in group 2, and 8 patients in group 3. IL-6 was significantly increased (cut-off value = 60 pg/mL [2]) in 62 cases (28 in group 1, 24 in group 2, and 9 in group 3). PCT was found to be elevated (cut-off value = 1.7 ng/mL [2]) in 62 cases: 29 in group 1,

Table 1. Incidence of risk factors of NS among the studied groups

Risk factor	Group 1 (n = 34)	Group 2 (n = 37)	Group 3 (n = 29)	P value	
Maternal fever	7	8	1	0.11	NS
PROM	4	6	0	0.21	NS
Preterm	12	6	2	0.007	Sig.
Low birth weight	11	17	9	0.88	NS
Instrumentation	22	13	8	0.003	Sig.

Sig.: significant; NS: not significant; PROM: rupture of the membrane > 18 hours; preterm: less than 37 weeks; low birth weight: less than 2,500 gram; Instrumentation included central line, IV cannula, urinary catheter, artificial ventilation, and naso-gastric tube.

Table 2. Comparison between results of blood culture, PCR, hs-CRP, IL-6, PCT, and hematological parameters among the studied groups

Studied parameter	Group 1(n = 34)	Group 2 (n = 37)	Group 3 (n = 29)	P value
Blood culture (+ve)	25	0	0	< 0.001 HS
PCR (+ve)	32	0	0	< 0.001 HS
hs-CRP*				
Positive	30 (88.2%)	35 (94.6%)	8 (27.6%)	< 0.001 HS
Negative	4 (11.8%)	2 (5.4%)	21 (72.4%)	
IL-6**				
Positive	28 (82.4%)	24 (64.7%)	9 (31.0%)	< 0.001 HS
Negative	6 (17.6%)	13 (35.3%)	20 (69.0%)	
PCT***				
Positive	29 (85.3%)	31 (83.8%)	2 (6.9%)	< 0.0001 HS
Negative	5 (14.7%)	6 (16.2%)	27 (93.1%)	
WBCs $X10^9$/L (M±SD)	10 ± 1.3	9.2 ± 5.1	10 ± 4.3	> 0.05 NS
Neutophils (%)(M ± SD)				
Total	55 ± 6.6	48 ± 9.3	51 ± 2.9	> 0.05 NS
Band cells	10 ± 4.1	12 ± 1.3	4 ± 2.5	< 0.05 Sig.
Platelets $\times10^9$/L (M ± SD)	169 ± 20.3	127 ± 12.3	145 ± 55.0	< 0.05 Sig.

HS: highly significant; Sig.: significant; NS: not significant; *cut-off value: 2.5mg/L; **cut-off value: 60 pg/mL ***cut-off value: 1.7 ng/mL [2]

Table 3. Results of PCR, hs-CRP, IL-6, and PCT in relation to the isolated organisms from blood of the studied groups

Isolated organisms by blood culture	PCR (+ve)	hs-CRP (M ± SD)	IL6 (M ± SD)	PCT (M ± SD)
*Klebsiella*spp. (n = 7)	7	17 ± 2.3	93 ± 4.7	13 ± 1.5
Escherichia coli (n = 5)	5	16.5 ± 4.2	102 ± 2.3	11 ± 3.3
*Enterobacter*spp. (n = 2)	2	17 ± 1.3	99 ± 2.5	14 ± 1.1
Serratiamarcescens (n = 1)	1	14.4 ± 0	101 ± 0	12.5 ± 0
*Acinetobacter*spp. (n = 1)	1	15 ± 0	98 ± 0	10.3 ± 0
Group B streptococcus (n = 3)	3	12 ± 1.5	80 ± 4.4	9 ± 1.1
CoNS (n = 3)	2	10 ± 1.3	79 ± 2.3	8.8 ± 2.3
Enterococcus (n = 1)	1	11.4 ± 0	82 ± 0	11 ± 0
Staphylococcusaureus (n = 2)	1	10 ± 2.4	82 ± 1.2	10 ± 1.2
P value	0.12 NS	0.15 NS	0.07 NS	0.10 NS

NS: not significant;CoNS: coagulase-negative staphylococci

Table 4. Sensitivity, specificity, and predictive values of the studied tests

	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
Blood culture	35.2	93.5	92.5	38.6
PCR	39	80.5	78.1	42.6
CRP	91.1	72.4	94.2	77.7
IL-6	63.6	69	75.6	55.5
PCT	72.5	90	93.5	71

31 in group 2, and only 2 cases in group 3. Sensitivity, and specificity of different methods are shown in Table 4.

Discussion

Neonatal sepsis is still one of the major causes of morbidity and mortality among newborns in developing countries. It is considered a life-threatening clinical emergency that necessitates urgent diagnosis and treatment [16-18]. The causative organisms of NS vary in developed and developing countries. In this study, Gram-negative organisms such as *Klebsiella* (seven isolates) and *E coli* (five isolates) were the most common causative organisms, followed by GBS (three isolates). Our results are consistent with other studies from Pakistan [19]. The predominance of Gram-negative organisms may be attributed to the current efforts toward maternal intra-partum antimicrobial prophylaxis, which significantly reduced the rates of GBS infection. Moreover, newborns most probably acquire these Gram-negative rods from the vaginal and fecal flora of the mother and the environment during delivery. On the other hand, other investigators found that CoNS was the most common causative organism of NS [20]. However, the mortality rate due to CoNS was lower than that due to Gram-negative bacilli. Similarly, Simonsen *et al.* [21] reported that while GBS was the most common etiologic agent of NS, *E.coli* was the most common cause of mortality.

Since sepsis is a systemic inflammatory response to infection, blood culture is still considered the gold standard method for diagnosis of NS [22]. In this study, only 25 out of 100 neonates who were suspected of having NS had positive blood culture. This low sensitivity (35.2%) may be attributed to inoculation of only 0.5 mL of blood or to the fact that about 60%–70% of infants had a low level of bacteremia. For optimal results, blood culture requires about 6 mL of blood, which is not feasible [23,24]. Maternal intra-partum antibiotic prophylaxis and small blood volume collections from infants have been considered as reasons for the lack of confidence in negative culture results [14]. PCR has been used to diagnose different pathogens (bacteria, viruses, and protozoa). Therefore, amplifying the DNA region common to all bacteria could represent an optimal method for diagnosis of bacterial sepsis [18]. In this study, 16S rDNA PCR assay was compared to blood culture as a tool in evaluating early-onset NS. Of 100 neonates screened for sepsis, 32 were positive by PCR. Compared to culture, PCR revealed higher sensitivity (42.6%) but lower specificity (80.5%); however, PCR failed to be positive in two samples obtained from blood culture-positive infants, and was positive in seven samples that were negative in blood culture. PCR combined with blood culture results revealed

bacteria in 34% of the patients diagnosed with NS. Similar results were reported by Reier-Nilsen *et al.* [2], who compared blood culture and PCR results for diagnosis of bacterial proven sepsis. On the other hand, Jordan *et al*. [14] reported different results; in their findings, 16S rDNA PCR demonstrated excellent specificity (97.5%) and negative predictive value (99.2%), but failed to detect a significant number of culture-proven cases.

Determination ofhs-CRP is one of the most commonly used laboratory tests for diagnosis of NS. As an acute-phase reactant, it plays a central role in the humoral immune response to infection. It is also useful for monitoring the response to treatment and guiding antibiotic therapy [25,26]. IL-6 is a pro-inflammatory cytokine produced by monocytes and macrophages activated by bacterial infection. IL-6 can be detected in blood earlier than CRP during the course of NS. Like most cytokines, IL-6 does not cross the placental barrier; therefore, its increased level may predict the possibility of NS during the first few hours of life [27]. In our study, hs-CRP level was found to be elevated in 71 neonates, with 91.1% sensitivity and 72.4% specificity. IL-6 was found to be raised in 63 neonates, with 63.6% sensitivity and 69% specificity. Also, IL-6 was mostly positive within 24 hours after onset of NS. Similar to our results, IL-6 was reported to have high sensitivity (76.9%), specificity (73.68%), positive predictive value (80%), and negative predictive value (70%) [28].

PCT is an acute-phase reactant produced both by hepatocytes and macrophages. It begins to rise four hours after exposure to bacterial infection; the PCT response is more rapid than that of CRP. Therefore, it is an attractive alternative for detection of NS because PCT levels remain high compared with IL-6. PCT is also useful in predicting severity of infection, response to treatment, and outcome [29]. In contrast to CRP, infants with other clinical conditions such as trauma, meconium aspiration, and hypoxemia have normal or minimal elevation in PCT [30,31]. In this study, it was found that PCT level was significantly elevated in 82 neonates suspected to have NS, with sensitivity of 72% and specificity of 90%. In other studies, similar results were demonstrated, where PCT sensitivity varied between 83%–100% and specificity varied between 70%–100% [32]. Our results support the findings of others [33,34] who suggested that PCT can be more accurate than CRP for diagnosis of NS.

Conclusions

The 16S rDNA PCR assay revealed higher sensitivity, and its combination with blood culture significantly increased the proved sepsis status among neonates suspected to have NS. None of the studied markers (hs-CRP, PCT, and IL-6) could be used individually to confirm or exclude the diagnosis of NS, and combination with other hematological markers and clinical observation is an essential issue. PCT was found to have greater value than hs-CRP and IL-6; however, further studies are needed to confirm these findings.

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Corresponding author

Emad M. Eed
Medical Laboratories Department, Faculty of Applied Medical Sciences
Taif University, 55 Abo Bakr St, Taif, Saudi Arabia
Phone: +966582765258
Email: dremadeed@yahoo.com

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