Statistical validity of interleukin-6 as a biomarker for the diagnosis of early-onset neonatal sepsis

Sriparna Basu,¹ Shashikant Dewangan,¹ Shampa Anupurva,² Ashok Kumar¹

¹Department of Pediatrics and

²Department of Microbiology, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India

Abstract

Use of empirical antibiotics in neonates with risk factors of early-onset neonatal sepsis (EOS) is a common practice. A laboratory parameter is needed to help in the accurate diagnosis of EOS to avoid unnecessary use of antibiotics. The aim of this prospective observational cohort study was to compare the statistical validity of cord blood interleukin-6 (IL-6) with conventional sepsis screening as an early diagnostic marker for EOS. Eighty-seven neonates with antenatal risk factors for sepsis were followed up for 72 h for the development of EOS. Cord blood was collected for measurement of IL-6 concentrations. Blood culture and conventional sepsis screening (total leukocyte count, absolute neutrophil count, C-reactive protein and micro-erythrocyte sedimentation rate) were sent for analysis soon after delivery. The study group comprised of symptomatic neonates with positive blood culture (n=36). An equal number of gestational-age matched asymptomatic neonates without risk factor of sepsis served as controls. Statistical validity of IL-6 was compared with sepsis screening parameters as the diagnostic marker for EOS. Gram negative organisms were the predominant cause of EOS. The most commonly isolated organism was *Acinetobacter baumannii*. The sensitivity and specificity of IL-6 with a cut-off value of 40.5 pg/mL and area under curve of 0.959 were 92.3 and 90.48%, respectively. In contrast, the sensitivity and specificity of different parameters of sepsis screening ranged from 37.5-68.75% and 47.95-57.35%, respectively. In conclusion, cord blood IL-6 can be used as a highly sensitive and specific early diagnostic marker of EOS at a cut-off concentration of 40.5 pg/mL.

Introduction

Sepsis is an important cause of neonatal morbidity and mortality in developing coun-

tries.¹ The World Health Organization estimates that 1 million deaths per year are due to neonatal sepsis and 42% of these deaths occur in the first week of life.² Although a large proportion of neonates are admitted with clinical suspicion of neonatal sepsis, diagnosis is at times difficult because of non-specific laboratory parameters. The practice of starting empirical antimicrobial therapy renders many neonates unduly susceptible to the side effects of antimicrobial agents, increases hospital costs, and promotes the development and spread of resistant bacterial strains.³

Blood culture is considered to be the gold standard in the etiological diagnosis of neonatal sepsis but pathogens can actually be identified only in a small subset of patients. Moreover, it takes at least 48-72 h to obtain a preliminary positive result according to conventional methods. At present, there is no single optimal diagnostic test to identify neonatal sepsis. Hematologic indexes and acute-phase reactants such as C-reactive protein (CRP) remain in widespread use but there are concerns about their reliability which largely emerge from the marked variations in their predictive accuracy.⁴ Therefore, there is the need for a single parameter which can detect neonatal sepsis with reasonable accuracy.

Contrary to late-onset neonatal sepsis (LOS), in which infection is acquired from the surroundings after birth, in EOS, infection is commonly acquired in utero resulting in the development of chorioamnionitis.⁵ Chorioamnionitis activates the fetal immune system leading to fetal inflammatory response syndrome (FIRS). In some neonates, FIRS is amplified and these neonates become symptomatic after birth with clinical manifestations suggestive of EOS. FIRS can be diagnosed by an increased number of proinflammatory cytokines in cord blood, of which interleukin-6 (IL-6) is one of the early mediators released in the circulation. Previous authors have documented high umbilical cord blood IL-6 concentrations in chorioamnionitis.⁶⁻⁸ In the present study, we compared the statistical validity of cord blood IL-6 concentrations with conventional sepsis screening as an early diagnostic marker of EOS.

Materials and Methods

Study population

The study was conducted in the Neonatal Unit of the Sir Sunderlal Hospital, Banaras Hindu University, India, over a period of one year and was approved by the Institute's Ethics Committee. The initial study group was made up of 87 newborns with one or more risk factors for development of EOS. Neonates were

Correspondence: Sriparna Basu, Department of Pediatrics, Institute of Medical Sciences, Banaras Hindu University, Varanasi, 221005, India.

E-mail: drsriparnabasu@rediffmail.com

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enrolled into the study, irrespective of their gestational age and sex, after the mother-infant dyads were screened for possible enrolment criteria. Risk factors for development of EOS were defined as:

- premature (<35 weeks of gestation) or prolonged (>18 h) rupture of membrane;
- clinical chorioamnionitis defined as intra-partum maternal fever (>37.8°C in the period from onset of labor to delivery) with two or more features: fetal tachycardia, uterine tenderness, malodorous vaginal discharge or maternal leukocytosis (leukocytes >15×10⁹/L).⁹

Cord blood was collected for measurement of IL-6 concentrations. Blood culture and conventional sepsis screening (total leukocyte count, absolute neutrophil count, C-reactive protein and micro-erythrocyte sedimentation rate) were sent for analysis soon after delivery. Symptomatic neonates with positive blood culture formed the final study group. An equal number of gestational-age matched asymptomatic neonates without risk factor of sepsis served as controls. Informed and written consent was obtained from the parents of all patients before their inclusion in the study.

Infants with perinatal asphyxia, intrauterine infections (TORCH, congenital syphilis, infective hepatitis, Human Immunodeficiency Virus), other systemic or metabolic diseases, and congenital malformations were excluded. Those who were discharged from hospital before 72 h of life were also excluded.

Clinical workup

Detailed antenatal and intranatal history, including demographic details of the mother and the family, were recorded in all neonates. Thorough clinical examination was made at birth to detect any systemic disease or congenital anomaly. Enrolled subjects were followed up for development of clinical signs of sepsis including: respiratory distress, temperature instability, feeding intolerance/abdominal distension/vomiting, neonatal hyperbilirubinemia, lethargy/irritability, apnea, bradycardia/tachycardia, arterial desaturations, hypotension/poor perfusion, hemorrhagic manifestations, convulsions or abnormal glucose homeostasis (documented by bedside Accucheck dextrostix), recorded every 6 h, for a period of 72 h after birth. Other investigations, such as laboratory estimation of blood glucose, serum electrolytes, bleeding and coagulation profile, liver and kidney function tests, arterial blood gas analysis, etc., were performed on clinical suspicion as and when indicated. Lumbar puncture and cerebrospinal fluid (CSF) analysis was made on clinical suspicion of meningitis. Neonates were managed according to the protocol of our unit. Progress during hospital stay and outcome were reported.

Collection of samples and laboratory analysis

After complete delivery, 10 mL of free flowing cord blood was collected for quantitative estimation of IL-6 in sterilized test tubes from the placental end of the umbilical cord without milking under aseptic conditions. Serum was separated immediately by centrifugation at 3,000 rpm and stored at -20°C until analyzed. IL-6 level was determined by Human IL-6 ELISA kit (Diacclone, GEN-PROBE Inc., San Diego, CA, USA) according to the manufacturer's instructions.

Single-site blood culture was taken within 30 min of birth from a peripheral vein, prior to starting antibiotics. A 20 mL blood culture bottle containing sheep brain heart infusion broth was inoculated with 1 mL of infant's blood under full aseptic conditions. Bacterial growth was noted after 48 h and then after seven days. Drug sensitivity was determined whenever bacterial growth was noted. Sepsis screening, including total leukocyte count (TLC), absolute neutrophil count (ANC), serum C-reactive protein (CRP), and micro-erythrocyte sedimentation rate (μ ESR), was performed in all infants between 12-24 h after delivery from peripheral venous blood. TLC and ANC were calculated from complete blood count (CBC), carried out on a Wipro diagnostic three chamber CBC machine. Quantitative serum CRP was assessed by nephelometry using the Orion Diagnostica kit, Finland. Micro-ESR was performed in a heparinized capillary tube after maintaining it vertical for 1 h.

Statistical analysis

The statistical program SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis. Sensitivity, specificity, positive likelihood ratio (PLR) and negative likelihood ratio (NLR) were calculated at selected cut-off values for IL-6 and sepsis screening parameters. Receiver operating characteristic (ROC) curve analyses with measurement of area under the curve (AUC) were performed to identify the appropriate cut-off values. $P < 0.05$ was considered statistically significant.

Results

A total of 36 newborns (25 males and 11 females) were diagnosed as EOS on the basis of development of clinical signs of sepsis within 72 h and positive blood culture by conventional methods at 48 h. Baseline parameters of cases and controls were comparable. Mean birth weight of cases and controls was 1632.8 ± 545 vs. 1585.2 ± 632 g, respectively ($P > 0.05$) and mean gestational age was

33.4 ± 2.8 vs. 32.8 ± 2.5 weeks, respectively ($P > 0.05$). There was no difference between the groups regarding maternal age, gravidity and parity, nature of antenatal care taken, obstetric complications, and mode of delivery. Both the groups showed male preponderance, none of them was intra-uterine growth restricted, and none of the babies required delivery room resuscitation.

Organisms grown in blood culture are summarized in Table 1. Gram negative organisms were predominant (91.7%). The most commonly isolated organism was *Acinetobacter baumannii* (27.8%), followed by *Klebsiella pneumoniae* (22.2%) and *Pseudomonas aeruginosa* (19.4%). Gram positive organisms (Staphylococci) were isolated in only 3 cases (8.3%).

Validity indexes of IL-6 and sepsis screening parameters are listed in Table 2. Cut-off values for the parameters of positive sepsis screening were defined as: $TLC \leq 5 \times 10^9/L$, $ANC \leq 1.8 \times 10^9/L$, $CRP \geq 10$ mg/L, and μ -ESR ≥ 10 mm in the first hour according to the protocol of our unit. The cut-off value of 40.5 for IL-6 was determined using ROC curve analysis (Figure 1). Area under curve of IL-6 was 0.959. Sensitivity, specificity, PLR and NLR of IL-6 at the cut-off value of 40.5 pg/mL were 92.3%, 90.48%, 9.69 and 0.08, respectively. Sensitivity, specificity, PLR and NLR of other parameters of sepsis screening is shown in Table 2. Sensitivity ranged individually from 37.5 to 68.75%, specificity from 47.95 to 57.35%, PLR from 1.61 to 1.27, and NLR from

Figure 1. Receiver-operating-characteristic (ROC) curve for interleukin-6. Area under curve 0.959.

Table 1. Organisms grown in blood culture in sepsis group (n=36).

Organism	N. isolated (%)
<i>Acinetobacter baumannii</i>	10 (27.8)
<i>Klebsiella pneumoniae</i>	8 (22.2)
<i>Pseudomonas aeruginosa</i>	7 (19.4)
<i>Escherichia coli</i>	5 (13.9)
<i>Citrobacter</i>	3 (8.3)
<i>Staphylococcus aureus</i>	2 (5.6)
<i>Staphylococcus epidermidis</i>	1 (2.8)

Table 2. Statistical validity indices for interleukin-6 and different parameters of sepsis screen.

Parameters	Cut off	Sensitivity (%)	Specificity (%)	Positive likelihood ratio	Negative likelihood ratio
Interleukin-6 (pg/mL)	$\geq 40.5^*$	92.3	90.48	9.69	0.08
TLC ($\times 10^9/L$)	≤ 5	55.5	56.2	1.27	0.79
ANC ($\times 10^9/L$)	≤ 1.8	37.5	49.3	1.36	1.2
CRP (mg/L)	≥ 10	68.75	57.35	1.61	0.96
μ ESR (mm in 1 st hour)	≥ 0	66.67	47.95	1.28	1.82
Sepsis screen positive for any 2 parameters	-	80	49.3	1.58	0.41

*Cut-off value of 40.5 for IL-6 was determined by using ROC curve analysis. TLC, total leukocyte count; ANC, absolute neutrophil count; CRP, serum C-reactive protein; μ ESR, micro-erythrocyte sedimentation rate.

1.82 to 0.79. Individually, CRP had the highest predictive accuracy among the sepsis screening parameters (sensitivity 68.75%, specificity 57.35%, PLR 1.61, and NLR 0.96). ANC showed minimum predictive accuracy (sensitivity 37.5%, specificity 49.3%, PLR 1.36, and NLR 1.2). When these two parameters were combined, sensitivity, specificity, PLR and NLR were 80%, 49.3%, 1.58 and 0.41, respectively.

Discussion

In this study, we compared the statistical validity of IL-6 with conventional sepsis screening parameters as an early diagnostic marker of EOS. We only included those cases of EOS with positive blood culture to avoid any problems in diagnosis. Males were predominant, and the majority of our study neonates were preterm and low birth weight. Predominant causes of EOS were Gram negative organisms except for 3 cases of *Staphylococcal* sepsis. The most commonly isolated organism was *Acinetobacter* (27.8%) followed by *Klebsiella* (22.2%) and *Pseudomonas* (19.4%). Two recent reports from eastern and southern India reported *Klebsiella* to be the most common organism grown in blood culture (48 of 131 and 29 of 39, respectively).^{10,11}

We found elevated umbilical cord blood IL-6 concentrations to be a better diagnostic tool compared to conventional sepsis screening in neonates who subsequently developed blood culture positive EOS. IL-6 is an inflammatory cytokine produced by a variety of cells, such as granulocytes, monocytes, lymphocytes, endothelial and some epithelial cell types, in response to bacterial infection. A wide variety of inflammatory mediators induce the release of IL-6 which include tumor necrosis factor alpha, IL-1, endotoxins, etc.¹² IL-6 is crucial for the initiation of an innate immune response and propagation of acquired immune responses through induction of T-cell activation and proliferation, as well as B-cell differentiation. It stimulates bone marrow and coagulation cascade, and furthermore, as a potent pyrogen, it stimulates the synthesis of a wide spectrum of acute phase proteins in human hepatocytes.¹² Jarvis *et al.* have demonstrated higher concentrations of IL-6 in umbilical cord blood at birth in the presence of chorioamnionitis and funisitis as the hallmark of FIRS.¹³ Several studies have demonstrated an early rise in IL-6 in neonatal sepsis.^{14,15} IL-6 has been shown to be rapidly released in response to infection, with peak serum levels within 2 h, often before the onset of clinical symptoms and before routine laboratory tests, such as measurement of high-sensitivity CRP (hsCRP), result positive¹⁶

We recorded IL-6 sensitivity and specificity of 92.3 and 90.48%, respectively, with a cut-off value of 40.5 pg/mL. Different authors have used different cut-off values of IL-6 for the diagnosis of EOS. Nishimaki *et al.*⁸ have used a cut-off of 54.7 pg/mL. Values as low as 10 pg/mL have been used by Martínez Nadal *et al.*,¹⁷ whereas Smulian *et al.*¹⁸ have used a cut-off of 25 pg/mL. A correlation between the severity of acute chorioamnionitis and umbilical venous IL-6 concentrations has also been documented by several authors. Kashlan *et al.* found that significantly elevated IL-6 concentrations with moderate to severe acute chorioamnionitis, acute deciduitis, and chorionic plate vasculitis. Clinically suspected or proven neonatal sepsis correlated with IL-6 elevations; a 30 pg/mL cut-off gave 95% sensitivity and 78% specificity, and a 100 pg/mL cut-off resulted in 84% sensitivity and 91% specificity.¹⁹ On the other hand, Khassawneh *et al.* found the cut-off value of 18.2 pg/mL to be associated with 87% sensitivity and 50% specificity.²⁰ In the study carried out by Schefold *et al.*, the sensitivity and specificity of IL-6 was found to be 82.6 and 86.5%, respectively.¹⁶

Hematologic markers which increase in response to infection include microscopic examination of the blood for white blood cells: total and differential leukocyte count, absolute neutrophil count, and immature to total neutrophil (I:T) ratio. Different authors found significant heterogeneity in the predictive value of hematologic indexes of sepsis screen. The possible sources of heterogeneity were: population, age, whether the subjects were at term or preterm, methodological quality, different leukocyte indexes, different cut-off values, and interpretation of test results by different laboratory observers. The majority of the studies have demonstrated low detection sensitivity for neonatal infection. Even the combination of all factors failed to reach an appropriate sensitivity and specificity in EOS.²¹⁻²³

The most commonly used acute phase reactant is CRP which has been widely studied as a diagnostic tool in neonatal sepsis and also as an indicator of response to therapy. However, CRP takes 12-24 h to increase to measurable levels; it has a very long half-life and it takes 5-7 days to normalize after eradication of the infectious agent.²⁴ Several authors have reported that CRP can not be used as a good predictor of neonatal sepsis alone.²⁵⁻²⁷

Sepsis screening did not perform well in our study with sensitivity of 80% and NLR of 0.41. Kudawla *et al.* reported a sensitivity of 48.3%, specificity of 70%, PLR of 1.61 and NLR of 0.738.²⁸ We have taken a relatively higher cut-off level of CRP (10 mg/L) to diagnose EOS. The study of Khassawneh *et al.* showed that a CRP of over 4 mg/L could give the best cut-off value with 95.7% sensitivity, 88.9% specificity,

78.6% PPV and 98% NPV.²⁰ The authors concluded that this CRP cut-off value offered the best single available test to predict or exclude Gram negative neonatal infections.

However, this study was not without limitations. Sample size was small because we strictly included only blood culture positive cases. The blood culture positivity rate in our unit has remained 30-35% over the last five years. We could not measure I:T ratio because we did not have the necessary facilities. Moreover, because of financial constraints, we could not measure multiple inflammatory cytokines to increase the specificity of the diagnostic tests, and we could not make serial measurements of IL-6 concentrations, although IL-6 kinetics could have been a better parameter to use to assess its diagnostic utility.

In conclusion, Gram negative organisms were the predominant cause of EOS in our study. Interleukin-6 concentrations in umbilical cord blood are elevated in chorioamnionitis. Umbilical cord blood IL-6 concentrations were found to be a highly sensitive and specific early diagnostic parameter at a cut-off concentration of 40.5 pg/mL in preterm, low birth weight neonates with EOS. Conventional sepsis screening markers, including hematologic indices and C-reactive protein, are not reliable early markers for early-onset neonatal sepsis.

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