Utility of cytokine, adhesion molecule and acute phase proteins in early diagnosis of neonatal sepsis

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Abstract

Background and Aim: Neonatal infection, including bacterial sepsis, is a major health care issue with an annual global mortality in excess of one million lives. Therefore, this study aimed to evaluate the potential diagnostic value of C-reactive protein (CRP), E-selectin, procalcitonin (PCT), interleukins-6 (IL-6), and tumor necrosis factor-α (TNF-α) both independently and in combination for the diagnosis of neonatal sepsis in its earliest stages. Materials and Methods: A total of 320 subjects were included in this study. A prospective cross-sectional study was conducted among neonates admitted to Neonatal Intensive Care Unit at King Abdulaziz Medical City, Riyadh, KSA during January 2013 to August 2015, the study based on three study groups categorized according to clinical symptoms and blood culture result. Study groups include healthy control neonates (n = 80), clinical sepsis (CS) group (n = 80) with clinical signs of sepsis but their blood culture was negative, and sepsis group with clinical signs of sepsis and their blood culture was positive. Results: The study observed significant difference in plasma levels of CRP, IL-6, TNF-α, E-selectin, and PCT in patients group when compared with control group (P < 0.001). Furthermore, the levels are significantly different between patient groups including CS and neonatal sepsis group. Moreover, result observed significant difference in CRP and IL-6 in early onset sepsis (EOS) when compared with late onset sepsis (LOS) neonates (P < 0.001 and 0.01), respectively, while there were no significant difference in TNF-α, E-selectin, and PCT between EOS and LOS (P = 0.44, 0.27 and 0.24), respectively. Regarding biomarkers accuracy, the result showed that CRP has the best diagnostic accuracy with cutoff value of 3.6 mg/ml (sensitivity 78% and specificity of 70%). The best combination is shown with CRP and IL-6 in which sensitivity increases to 89% and specificity to 79%. Conclusion: It was concluded that infected new-born babies have a higher E-selectin, PCT, IL-6, TNF-α, and CRP compared with the neonates with CS and control. IL-6, TNF-α, and CRP should be measured in combination for more diagnostic accuracy in neonatal sepsis. Likewise, PCT should be investigated as a part of sepsis screening for all suspected neonates.

Key words: C-reactive protein, cytokines, diagnostic accuracy, early onset sepsis, E-selectin, late onset sepsis, neonatal sepsis, procalcitonin
INTRODUCTION

Bacterial sepsis and infection of neonates are considered major health care issues with an annual global mortality that exceeds one million lives. Indeed, the leading cause of mortality among infants in the 1st day of life is infection.[12] The rate of neonatal sepsis remains high in tertiary care Neonatal Intensive Care Unit (NICU) in Saudi Arabia and exceeds internationally reported rates.[8]

The neonatal sepsis is an infectious disease of various etiologies and is described as systemic inflammatory response syndrome associated with proven infection which decides degrees of inflammatory response and metabolic change.[8,3] The immune system of neonates is not completely developed, and thus they are more prone to bacterial infection. In neonatal period, innate immune cells including macrophages and dendritic cells (DCs) initiate an effective immune response. Cytokines produced by macrophages and DCs play an important role during host defense mechanisms in response to infectious pathogens.[6,7]

Blood culture test for bacteria often referred to as the gold standard is the most reliable diagnostic test of neonatal sepsis. However, it can take 48–72 h for the results to be released. As a result, treatment should often begin before the laboratory results are released. An additional challenge is the fact that the blood culture test can be false negative for one in five subjects with sepsis.[8] Thus, it is of critical importance to identify new biomarkers that will enable fast and reliable diagnosis using chemical and hematological scoring systems for sepsis in its earliest stages. An acute phase inflammatory marker such as C-reactive protein (CRP) does not accurately differentiate between the systemic inflammatory response and sepsis when measured individually.[10,11] Procalcitonin (PCT), peptide precursor of the hormone calcitonin, is an 116 amino acid protein secreted by parafollicular C-cells of thyroid gland in normal healthy individual. However, the levels usually increase during septicemia, pneumonia meningitis, and urinary tract infection.[11,12] This biomarker is also produced by other cells such as macrophage, and monocyte of various organs in conditions of severe bacterial infection and sepsis.[13]

E-selectin, also known as CD62 antigen-like family member E (CD62E), is a cell adhesion molecule expressed only on endothelial cells activated by various cytokines. The unique role of selectins is represented by the direct leukocyte interaction with vascular endothelium by binding to carbohydrate epitopes on leukocyte or on endothelial cells.[14]

Other biomarker is tumor necrosis factor-α (TNF-α) which produce mainly by macrophage and monocyte.

Release of TNF-α often occurs approximately 30 min after lipopolysaccharide injection, and the circulating level reaches the peak in approximately one and a half hour, with an estimated half-life of about 70 min.[15–17] TNF-α regulates the secretion of IL-1 β while high level of TNF-α is associated with severity of disease. There are evidence that TNF-α is found free in plasma concomitant with the appearance of clinical signs and symptoms of infection.[18–21]

Interleukin-6 (IL-6) is the most frequent studied cytokine in neonatal population. IL-6 is a cytokine with early response to infection, preceding the increase in CRP and followed by release of TNF-α. This cytokine is synthesized by mononuclear phagocytes, fibroblasts, endothelial cells, and trophoblast cells immediately after stimulation by microbial products.[17,22]

This study intends to evaluate the potential diagnostic value for common used biomarkers CRP, E-selectin, PCT, IL-6, and TNF-α both independently and in combination for the diagnosis of neonatal sepsis. In addition, the study aims to identify the optimal cutoff value for these biomarkers to assess whether these markers could be helpful in early discontinuation of unnecessary antibiotic in noninfected newborn.

MATERIALS AND METHODS

Study area

A prospective cross-sectional (hospital base) study was conducted among neonates admitted to NICU at King Abdulaziz Medical City (KAMC), Riyadh, KSA during January 2013 to August 2015.

Study population and sample size

Patients were selected based on the international criteria of center for disease control for diagnosis of neonatal sepsis as have postnatal signs of sepsis and a positive blood culture from sample of peripheral or central venous lines.[23]

The study based on three study groups is categorized according to clinical symptoms and blood culture results. Group I (control) is eighty healthy neonates who were born normally at KAMC without any abnormal signs or symptoms of infection. Group II is clinical sepsis (CS) that was recruited among eighty neonates who admitted to NICU suspected to suffer from neonatal infection due to the presence of clinical signs of sepsis but their blood culture was negative. Group III is subdivided into two subgroup. First is early onset sepsis (EOS) that includes eighty neonates admitted to NICU at KAMC with clinical signs of sepsis appeared in first or 2nd day of life, and...
their blood culture was positives. Second subgroup is late onset sepsis (LOS) that includes eighty neonates admitted to NICU at KAMC with clinical signs of sepsis appeared after 3 days of life, and their blood culture was positives.

**Inclusion criteria**

Neonates with three or more of the following clinical signs were selected in patient group: (1) Respiratory manifestations, (2) bradycardia, (3) hypotonia or seizures, (4) poor skin color or capillary filling time >2 s, and (5) irritability or lethargy. The symptoms were recorded by the resident neonatologist at NICU, KAMC.

**Exclusion criteria**

Neonates diagnosed with congenital malformations, congenital infections associated with the TORCH complex, and refusal of parents were excluded from this study.

**Sampling**

A total of 5 ml of venous blood sample was obtained at respective time and separated into two tubes. First tube was used for blood culture and second tube for analysis of CRP, IL-6, TNF-α, PCT, and E-selectin. Plasma was separated within 30 min of collection and stored at −20°C for analysis.

**Laboratory methods**

Plasma CRP, IL-6, TNF-α, and E-selectin were determined by sandwich enzyme-linked immunosorbent assay kit (Abcam®, Cambridge, UK) following the manufacturer instructions. Unknown samples, standards, and control were analyzed in duplicate and mean value was determined for each test. The assay uses two antigen-specific monoclonal antibodies that bind to respective analyte (as an antigen) at different binding sites. One of these antibodies was biotinylated specific monoclonal antibodies and the other was coated in microtiter plate wells. Streptavidin – horseradish peroxidase was used as enzyme conjugate that binds to secondary antibodies. 3,3',5,5'-tetramethylbenzidine substrate was then added to each well to produce color reaction. A stop solution was added to reaction and plate was read using VeraMax reader.

An immunoluminometric assay (LIAISON BRAHMS, Germany) was used for the specific measurement of PCT in serum (detection limit 0.10 ng/ml) following the instructions of the manufacturer. The assay uses two antigen-specific monoclonal antibodies that bind PCT (as an antigen) at different binding sites (the calcitonin and katabcalcin segments). One of these antibodies was luminescence-labeled (the tracer) and the other was coated on with magnetic particles (solid phase).

**Statistical analysis**

Descriptive and analyzing tests (Mann–Whitney rank-sum test, Student’s t-test, Kruskal–Wallis H-test, Chi-square test, Pearson correlation, and Spearman rank correlation) were performed using SPSS software 16 for Windows (Chicago, Illinois, USA).

The reliability of analytes concentration for the diagnosis of sepsis was calculated by receiver operating characteristics (ROC) curves. ROC index (sensitivity + specificity - 1) was used for determination of optimal cutoff values of the diagnostic tests in the different postnatal periods. Sensitivity, specificity, and the likelihood ratio of positive and negative results with a 95% confidence interval were calculated. Statistical significance was set at P < 0.05. Variables are presented as mean ± standard error of mean. For comparison of serum CRP, PCT, E-selectin, IL-1B, and TNF-α between the groups, Kruskal–Wallis test were employed followed by post-hoc tests.

**RESULTS**

Results of the present study include 320 neonates divided into three groups, age and sex were matched between the three groups and showed no significant difference (P = 0.9). The mean ± standard deviation of weight in control group was 3610 ± 483 g and 1944 ± 560 g for patients group with significant difference between them (P < 0.05).

**Clinical characteristics of patients groups**

Comparing the clinical finding of patients groups, unstable respiration signs was found to be 29 (23%), 48 (60%), and 16 (20%) in CS, EOS, and LOS group, respectively (P < 0.001). Cases with rash or skin infection signs was found to be 29 (23%), 48 (60%), and 16 (20%) in CS, EOS, and LOS group, respectively (P < 0.001). No significance difference was observed in tachycardia, bradycardia, hypoglycemia, acidosis, or jaundice between patients group (P > 0.05 for all). Detailed clinical characteristics of patients group are summarized in Table 1.

**Biomarkers in study population**

In reference to Mann–Whitney U-test, results of the present study observed significant increase in plasma levels of CRP, IL-6, TNF-α, PCT, and E-selectin in patients group compared to healthy control group (P < 0.001 for all). As shown in Table 2, Kruskal–Wallis H-test indicates a significant difference in all biomarkers between patients groups (P < 0.001) with high level of significant difference for CRP indicated by high H value (H = 124). Comparing cultured proved sepsis patients, CRP and IL-6

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**Table 1. Clinical characteristics of patients groups**

| Group     | Number of Patients | Percentage |
|-----------|--------------------|------------|
| Control   | 100                | 100%       |
| CS        | 100                | 100%       |
| EOS       | 60                 | 60%        |
| LOS       | 60                 | 60%        |

**Table 2. Biomarkers in study population**

| Biomarker | Control | CS | EOS | LOS | P-value |
|-----------|---------|----|-----|-----|---------|
| CRP       | 3.5     | 20 | 48  | 16  | <0.001  |
| IL-6      | 6.8     | 32 | 48  | 16  | <0.001  |
| TNF-α     | 2.5     | 30 | 48  | 16  | <0.001  |
| PCT       | 0.5     | 15 | 30  | 16  | <0.001  |
| E-selectin| 1.8     | 20 | 30  | 16  | <0.001  |

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showed a significant increase in LOS compared to EOS group ($P < 0.001$, $P = 0.01$, respectively). In contrast, no significant differences were found in TNF-α, E-selectin, and PCT between EOS and LOS group ($P = 0.44$, $0.27$ and $0.24$, respectively) [Table 3].

**Causative bacteria in sepsis groups**

Among 160 proved sepsis patients, *Escherichia coli* are isolated from 54 (68%) samples in EOS group and 18 (22%) samples in LOS group. Gram-positive Group B *Streptococcus* bacteria is associated with LOS in which 38 (48%) bacteria is isolated while only 5 (6%) are found in EOS group. Details in isolated bacteria are summarized in Table 4.

**Diagnostic accuracy of the biomarkers**

Table 5 summarizes sensitivity, specificity, and likelihood ratios for the biomarkers and best combination revealed after analysis. ROC curve was used to define the optimal cutoff value for the five biomarkers. Comparison of each test using optimal cutoff value showed higher index for CRP (0.85) with sensitivity of 78% and specificity of 70%. IL-6 showed higher sensitivity (83%) but specificity was 68%. Best specificity (70%) for individual biomarker was showed by three biomarkers (CRP, TNF-α, and PCT). However, E-selectin showed the least diagnostic accuracy with ROC index of 0.74, sensitivity of 73%, and specificity of 60%. Youden index was used to calculate best combination between these biomarkers. CRP and IL-6 showed the best combination at cutoff values of 3.6 ng/ml and 83 ng/ml, respectively. Sensitivity was increased to 89% and specificity to 79% with ROC index of 0.89. CRP and TNF-α combination yields higher specificity (85%) with sensitivity of 79%. Relatively same result revealed using CRP and PCT combination. ROC curves for the mentioned combination are shown in Figure 1 as it clearly indicates increasing the area under the curve (AUC) in combination of two biomarkers. Diagnostic accuracy of the biomarkers in solo and combination is shown in Table 5.

**DISCUSSION**

Neonatal sepsis evaluations with positive blood culture were diagnosed as culture proven neonatal sepsis according to National Healthcare Safety Network definitions of laboratory-confirmed bloodstream infection.[23] Infant with positive clinical score of sepsis was categorized as CS.[24] This includes infant with another infectious diagnosis that is not accompanied by positive blood culture, such as pneumonia, urinary tract infection and necrotizing enterocolitis. Most studies in neonatal sepsis suggest that age and sex have no effect in categorizing infected and noninfected infants and this was supported by the current study ($P > 0.05$).

One of the methodological limitations in this study is the lack of some clinical data such as gestational age and mode of delivery; however, the findings are strengthened by relatively large number of investigated neonates, the presence of positive blood culture in all septic neonates, investigation of both EOS and LOS, and minimizing the interference by collecting the samples before administration of antibiotics. Since clinical signs of sepsis are nonspecific,
diagnostic laboratory method is needed for early diagnosis, prognosis, and effective treatment.

Due to high mortality and morbidity among neonatal sepsis, laboratory markers with 100% sensitivity (all infected neonates have positive test) and negative predictive value (negative test accurately rule out the disease) are required.

It is well-known that several biochemical and immunological markers increased in the plasma during neonatal sepsis, such as increased CRP, IL-6, TNF-α, PCT, and E-selectin. Similarly, the results of the present study observe a significant increase in these biomarkers among proved-sepsis neonates compared to neonates with CS and health infants. The optimal cutoff value of the biomarkers for diagnosis of neonatal sepsis is not defined clearly, and there is divergence in many studies. Accordingly, we attempt to define the optimal cutoff value using ROC curve. The cutoff value of CRP, IL-6, TNF-α, E-selectin, and PCT is found to be 3.29 ng/ml, 705 ng/ml, 18.2 pg/ml, 168 ng/ml, and 2.7 ng/ml, respectively. These values were much closed to those recommended by manufactured in the package insert of each reagent kit (IL-6 <700, CRP <1.6, TNF-α <16, E-selectin <160, and PCT <3).

Among acute phase proteins, CRP is probably the most well-studied, but the findings regarding its diagnostic accuracy are mixed. CRP is synthesized in liver in response to inflammation and its secretion is stimulated by IL-1, IL-6, and TNF-α. The level increased 6–8 h after exposure to infection, reach peak level at 24–48 h, and return to normal overtime as inflammation resolved. Reviewing several studies in CRP, it has been reported that CRP level >1 mg/dl, as cutoff point, has sensitivity ranged between 70% to 93% and specificity ranged between 41% and 98%. In this study, the most appropriate cutoff point using ROC curve was found to be 3.6 ng/ml. Using this value, sensitivity was 78%, specificity 70%, and AUC 0.85. However, increased CRP level in CS group (mean 3.7 ng/ml) thought to affect the specificity. In addition, the increase of CRP level is rather slow during the first 12–24 h of infection and this may negatively affect the sensitivity. The moderate sensitivity and specificity in this study and many other studies strongly suggest that CRP individually will not be sufficient for the clinician to rely on it. TNF-α has the same behavior of IL-6 and it has a good specificity and sensitivity. This was clearly demonstrated in this finding by very close results in IL-6 and TNF-α in solo and combination. In contrast, CRP reaches the peak level relatively later, and hence it is more specific for confirming infection. Therefore, an abnormal high level of CRP with normal plasma level of IL-6 proposes recent infection 24–48 h.

PCT, as an acute phase protein, has been intensively investigated as inflammatory marker in neonatal sepsis. Many studies reported that PCT increased 4 h after exposure to endotoxin, reached peak level 6–8 h after infection, and remained in high level for at least 24 h. While PCT level is undetectable in healthy individual, the concentration increased gradually in relation to severity of inflammation in septic patients. As CRP, PCT synthesized by the liver and its production is stimulated by CRP and inflammatory mediator such as IL-6 and TNF-α. PCT remains in high level despite decrease of the stimulus mediator as inflammatory process is ongoing. In previous studies, sensitivity of PCT was ranged between 83% and 100% and specificity ranged between 70% and 100%. Hence, in this study, PCT was found to be in lower limit of accuracy (sensitivity 72 and specificity 70%). In addition, our results indicate less accuracy of PCT than CRP in the diagnosis of neonatal sepsis. However, Kocabas et al. reported that PCT is

### Table 4: Causative bacteria according to onset of sepsis

| Causative bacteria                  | EOS (n=80) (%) | LOS (n=80) (%) |
|-------------------------------------|----------------|----------------|
| *Escherichia coli* (Gram-negative) | 54 (68)        | 18 (22)        |
| *Hemophilus influenza* (Gram-negative) | 21 (27)       | 2 (3)          |
| Group B *streptococcus* (Gram-positive) | 5 (6)         | 38 (48)        |
| *Staphylococcus aureus*             | 0 (0)          | 22 (27)        |

EOS: Early onset sepsis, LOS: Late onset sepsis

### Table 5: Sensitivity, specificity and likelihood ratios biomarkers

| Cytokines | Cutoff | Sensitivity | Specificity % | ROC (95% CI) | LR * | LR * |
|-----------|--------|-------------|---------------|--------------|------|------|
| CRP       | 3.6    | 78          | 70            | 0.85 (0.81-0.89) | 2.29 | 0.44 |
| IL-6      | 705    | 83          |               |              | 2.51 | 0.28 |
| TNF-α     | 18.1   | 69          | 70            | 0.74 (0.68-0.79) | 1.81 | 0.45 |
| E-selectin| 168    | 73          | 60            | 0.81 (0.75-0.86) | 2.35 | 0.34 |
| PCT       | 2.74   | 72          | 70            | 0.89 (0.85-0.93) | 2.41 | 0.38 |
| CRP/IL-6  | 89     | 79          |               | 0.88 (0.84-0.92) | 2.36 | 0.37 |
| CRP/PCT   | 78     | 85          |               | 0.85 (0.81-0.88) | 2.12 | 0.11 |
| IL-6/PCT  | 85     | 74          |               | 0.88 (0.84-0.92) | 2.01 | 0.32 |
| TNF-α/CRP | 79     | 85          |               | 0.85 (0.80-0.89) | 2.41 | 0.29 |
| TNF-α/IL-6| 87     | 74          |               | 0.83 (0.79-0.88) | 2.60 | 0.45 |

ROC: Receiver operating characteristic, CI: Confidence interval, CRP: C-reactive protein, IL-6: Interleukin-6, PCT: Procalcitonin, TNF-α: Tumor necrosis factor-α, LR: Likelihood ratio
Figure 1: Receiver operating characteristics curve for biomarkers in solo and combination. (A1–C1) receiver operating characteristics curves for IL-6 and C-reactive protein individually and in combination, (A2–C2) receiver operating characteristics curves for procalcitonin and C-reactive protein individually and in combination, (A3–C3) receiver operating characteristics curves of C-reactive protein and tumor necrosis factor-α individually and in combination
more accurate than CRP. Other studies reported that PCT is not better marker than CRP. Diversity in the results suggests that physiological alterations observed in the 1st and 2nd day of life and the effect of prenatal and postnatal antibiotic administration will affect the PCT level and functions as confounder in relation between PCT and infection. Moreover, in the current finding, we observe no significant difference in PCT level between EOS and LOS and this suggests that PCT level does not affect by age at which onset of infection occurs.

Chiesa et al., reported that increased PCT level in neonatal sepsis is caused by perinatal events rather than infection induction. The accuracy of PCT depends on birth weight in which it shows more accuracy below 1500 g birth weight. Other study by López Sastre et al. reports that PCT is not sufficient when measured individually in case of hospital-acquired neonatal sepsis. It has been cautioned that PCT could not serve as individual marker for neonatal sepsis and should be a part of full sepsis work up.

IL-6 produced rapidly in the beginning of neonatal sepsis course. This rapid elevation will be diminished shortly because of short half-life of IL-6. This specific property makes IL-6 useful as a very early indication for neonatal sepsis, but due to short half-life, clinician could not rely on IL-6 alone. A significant increase was observed in LOS when compared to EOS. This finding is supported by several studies and this emphasizes the fact that IL-6 has a natural fluctuation immediately after postnatal period.

Best combination revealed by this study has been found in IL-6 (early and sensitive marker) and CRP (late and specific marker) in which AUC was the highest (0.89) with 89 and 79% for sensitivity and specificity, respectively. Similar results were reported by several studies in investigating diagnostic markers of neonatal sepsis.

**CONCLUSION**

There is no doubt that inflammatory biomarkers such as cytokines, adhesion molecules, and CRP are increased in neonatal sepsis. However, these biomarkers should be used in proper pattern for best diagnosis to increase positive predictive value. PCT determination should be included in full sepsis screening test for all suspected neonates. A combination of IL-6, TNF-α, and CRP yield best diagnostic accuracy when used in two occasions during the course of the disease.

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**Conflicts of interest**

There are no conflicts of interest.

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