Validity of Interleukin-18 plus CRP Measurement in the Early Diagnosis of Sepsis Neonatorum

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Background: Over the last decade a variety of laboratory tests have been developed to enhance the early and accurate diagnosis of sepsis neonatorum. However, none of these tests has been found to be absolutely reliable in detecting all septic neonates. Objective: To test the validity of circulating interleukin-18 (cIL-18) plus serum C-reactive protein (CRP) for the diagnosis of sepsis neonatorum, in term infants admitted to the neonatal intensive care unit (NICU) of Zagazig University Hospitals, during year 2005. Study design: Twenty – four neonates with positive blood cultures were selected from 60 neonates with clinically suspected sepsis. Their ages ranged from 0.3 to 25 days (X ± SD: 14.2 ± 7.1). In addition, 14 gestational age (GA) -, chronologic age – and sex – matched healthy neonates served as a control group. Results: Forty percent of neonates with clinically suspected sepsis proved to have positive blood cultures, E. coli being the most significant isolate (62.5%). Rise of serum CRP and cIL-18 is highly significantly associated with culture-proven neonatal sepsis. Meanwhile, other tests were nonsignificant associates. Using ROC curve analysis, cIL-18 displayed a sensitivity and a specificity of 91.7% and 85.7%, and CRP displayed a sensitivity and a specificity of 72% and 100%, respectively. When both tests were used, combined, the reported sensitivity and specificity accounted for 100%, for each. Conclusion: The combined use of cIL-18 and serum CRP is valuable in the early and accurate diagnosis of sepsis neonatorum. Key wards: sepsis-neonatal-proteic marker.

INTRODUCTION

Despite extensive medical advances over the past two decades, the reported number of sepsis continues to increase at an alarming annual rate because of limited progress in diagnostic methods.(1)

Infants, who present with clinical signs of sepsis neonatorum are evaluated with a variety of diagnostic tests, commonly referred to as a sepsis work-up, and treatment with broad-spectrum antibiotics is initiated until a definitive diagnosis [isolation of microorganism(s) from blood culture(s)] can be made.(2) However, the sole use of blood cultures, for diagnosis, has a number of limitations; 1) It may take 1 to 3 days to obtain culture results, thus delaying the definitive diagnosis; 2) the sensitivity in diagnosis in very low (9% - 35%), and 3) the sensitivity may be further impaired by exposure to intrapartum antibiotic prophylaxis (IAP), delaying the onset of clinical signs and symptoms of infection, and further complicating the expedient definitive diagnosis of early – onset sepsis neonatorum.(3)

IL-18, a potent gamma interferon (IFN-γ) inducer, is a member of the IL-1 family of ligands, produced mainly as a precursor protein (24 Kda) that requires proteolytic activation by an IL-1 β-converting enzyme to liberate the 18-Kda mature active protein. IL-18 mRNA is expressed in a wide range of cells, including T cells and B cells.(4) Recent studies investigated the role of IL-18 in the host response to infection. In its capacity as an IFN-γ inducing factor, IL-18 has an important role in innate immunity and acquired immunity.(5) The usefulness of this marker has been reported in several studies, including the differentiation of sepsis from severe injury. However, no information has been reported pertaining IL-18, in neonatal sepsis.(1)

CRP, a nonspecific acute-phase protein that rises in response to infectious and non-infectious inflammatory processes, is an endo-genous vitamin K-dependent anticoagulant which plays a major role in the down-regulation of the pro-coagulant arm. Endothelial damage, during sepsis impairs the endothelium dependent activation of protein C, thus shifting the balance towards thrombosis. This shift may contribute to the development of sepsis-related multi-organ failure.(6) This study aimed to test the validity of combined CRP and cIL – 18 measurement
SUBJECTS AND METHODS

Subjects:
Sixty sick neonates of GA ranging between 36 and 40 weeks (X ± SD : 38 ± 1.8), with symptoms and signs suggestive of sepsis, according to Stoll and Bang et al (3,7), were enrolled in the study. In addition, 14 GA-, chronologic age-and sex-matched apparently healthy neonates, with normal pregnancy and delivery, served as a control group. Written consents were obtained from parent(s) or care-giver(s) of infants, before inclusion in the study.

Methods:
All neonates were subjected to:
A) History-taking and physical examination, including assessment of GA, according to Narayanan (8).
B) Total white blood cell count (WBCs) and percent of band cells.
C) Blood culture, according to Shang et al (9).
D) Measurement of serum CRP, according to Weinberg and Powell (10).
E) Serum IL-18 assay, using human IL-18 ELISA Kit.

The assay will recognize both natural (pro and mature) human IL-18. This kit has been configured for research use only.

Principle of the test:
The IL-18 kit is a solid phase sandwhich ELISA. A polyclonal antibody specific for IL-18 has been coated onto the wells of the micor – titer strips provided. Samples, including standards of known IL-18 concentrations are pipetted into these wells. During the first incubation, the IL-18 antigen and a biotinylated polyclonal antibody specific for IL-18 are simultaneously incubated. After washing, the enzyme (streptavidin- peroxidase) is added. After incubation and washing to remove all the unbound enzyme, a substrate solution which is acting on the bound enzyme is added to induce a coloured reaction product. The intensity of this coloured product is directly proportional to the concentration of IL-18 present in the sample.

Assay method:
a) Before use, mix all reagents thoroughly without making foam.
b) Determine the number of micro-well strips required to test the desired number of samples, plus appropriate number of well needed for running blanks standards and control. Each sample, standard, blank and control samples should be assayed in duplicate. Remove sufficient microwell strips from the pouch.
c) Add 100 µl of appropriate standard diluent (x10 distilled water) to standard wells B1, B2, C1, C2, D1, D2, E1, E2, F1, F2. Pipette 200 µl of standard into wells A1 and A2. Transfer 100 µl from A1 and A2 to B1 and B2 wells. Mix the contents by repeated aspirations and ejections. Take care not to scratch the inner surface of microwells. Repeat this procedure from the wells B1, B2 to wells C1, C2 and from wells C1, C2 to D1, D2 and so on creating two parallel rows of IL-18 standard dilutions ranging from 200 to 62.5 pg/ml. Discard 100 µl from the content of the last microwells used (F1, F2). Alternatively these dilutions can be done in separate tube and diluted standard pipetted directly into wells.
d) Add 100 µl of appropriate standard diluent to the blank wells (G1 – G2).
e) Add 100 µl of sample to sample wells.
f) Preparation of biotinylated anti-IL-18.
g) Add 50 µl of diluted biotinylated anti-IL-18 to all wells.
h) Cover with a plate cover and incubate for 3 hours at room temperature (18°C – 25°C).
i) Remove the cover and wash the plate.
j) Prepare streptavidin-HRP solution before use.
k) Distribute 100 µl of streptavidin-HRP solution to all wells, including blank wells.
l) Cover and incubate 30 minutes at room temperature.
m) Remove the cover and empty wells. Wash microwell strips according to step i). Proceed immediately to the next step.
n) Pipette 100 µl of ready-to-use tetra-methyl benzidine (TMB) substrate solution into all wells, including the blank wells and incubate in the dark for 12-15 minutes at room temperature. Avoid
direct exposure to light by wrapping the plate in aluminium foil.

Incubation time of the substrate solution is usually determined by the ELISA reader performances: many ELISA readers record absorbance only up to 2.0 optical density (OD). The O.D. values of the plate should be monitored and the substrate reaction stopped before positive wells are no longer properly readable (maximum 20 minutes).

The enzyme-substrate reaction is stopped by quickly pipetting 100 µl of H2SO4 stop reagent into each well, including the blank wells, to completely and uniformly inactivate the enzyme. Results must be read immediately after the addition of H2SO4, or within one hour, if the microwell strips are stored at 2-8°C in the dark.

Read absorbance of each well on a spectrophotometer using the primary wavelength 620nm (610nm to 650 nm is acceptable) as the reference wavelength.

Data analysis:
Generate a linear standard curve by plotting the average absorbance on the vertical axis versus the corresponding IL-18 standard concentration on the horizontal axis. The amount of IL-18 in each sample is determined by extrapolating OD values to IL-18 concentrations using the standard curve (Figure 1).

Figure 1: Typical IL-18 standard curve ranging from 62.5 to 2000 pg/mL

F) Statistical Analysis
Data were collected and checked to an Epi-Info file (version 6.1) computer package. Data were expressed as mean ± standard deviation (X ± SD) in quantitative variables, number and percentage for qualitative variables. Student “t” test and chi square (χ²) were used for analysis. For the above mentioned tests, the threshold of significance is fixed at 5% level (p-value) 11).

Receiver of operating characteristics (ROC) curve analysis:
The diagnostic performance of a test (cIL-18 and serum CRP), or its ability to discriminate diseased cases from normal ones is evaluated using ROC curve analysis. When we consider the results of a particular test in two population, one with a disease and the other without the disease, we will rarely observe a perfect separation between the two groups. Therefore, a particular cut-off point discriminate between the two populations 12).

The reference cut-off point reported for cIL-18, is 188 pg/ml. Neonates having values > 188 pg/ml are considered septic 13).

Meanwhile, the reference cut-off point reported for serum CRP is 10 mg/L 14).

Validity tests 15)
The following statistics can be defined:

Sensitivity: Probability that a test result will be positive when the disease is present (true positive rate, expressed as a percentage) = true positive / (true positive + false negative).

Specificity: Probability that a test result will be negative when the disease is present (true negative rate, expressed as a percentage) = true negative / (true negative + false positive).

RESULTS
Out of 60 infants, suspected to have clinical neonatal sepsis, 24 (40%) proved to have positive blood cultures, 15 isolates (62.5%) revealed E. coli, 5 (20.8%) revealed group B Streptococci (GBS) and 4 (16.7%) of isolates revealed Staphylococci (Staph), with highly significant difference, p<0.01.

Maternal events in the last trimester and during labor are significantly associated with culture–proven neonatal sepsis. Meanwhile, clinical neonatal criteria significantly associated with culture-proven sepsis include drowsy / unconsciousness, abnormal / weak cry, sucking weak or stopped (>8 hours), limbs limp, hypothermia, oliguria / anuria, diarrhea/ vomiting / abdominal distension,
tachypnea / grunt / chest indrawing, intermittent apnea/ cyanosis, and hemorrhage / seizures. Meanwhile, fever (≥ 38°C), cough / nasal discharge, and umbilical/skin sepsis were not significant associates (p>0.05). Table 1.

Rise of CRP is significantly associated with culture – proven neonatal sepsis (P<0.01). Increased circulating IL-18 levels show a very highly significant association with culture – proven neonatal sepsis, P<0.001. Tests other than CRP and IL-18 proved to be non significant for the diagnosis of neonatal sepsis, Table 2.

Table 1: Clinical criteria to identify neonatal sepsis among 24 infants proven to have sepsis versus control infants.

<table>
<thead>
<tr>
<th>Symptom/sign</th>
<th>Septic neonates n= 24</th>
<th>Control neonates n = 14</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Maternal, no &amp; (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In the last trimester *</td>
<td>7 (29.2)</td>
<td>2 (14.3)</td>
<td>S</td>
</tr>
<tr>
<td>During labor **</td>
<td>4 (16.7)</td>
<td>1 (7.1)</td>
<td>S</td>
</tr>
<tr>
<td>During 0-28d postpartum ***</td>
<td>5 (20.8)</td>
<td>3 (21.4)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Neonatal, no &amp; (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drowsy/unconscious</td>
<td>2 (8.3)</td>
<td>-</td>
<td>S</td>
</tr>
<tr>
<td>Cry abnormal/weak/or stopped</td>
<td>3 (12.5)</td>
<td>1 (7.1)</td>
<td>S</td>
</tr>
<tr>
<td>Sucking /reduced or stopped</td>
<td>3 (12.5)</td>
<td>1 (7.1)</td>
<td>S</td>
</tr>
<tr>
<td>Limbs became limp</td>
<td>2 (8.3)</td>
<td>-</td>
<td>S</td>
</tr>
<tr>
<td>Fever 38.5 °C</td>
<td>4 (16.7)</td>
<td>2 (14.3)</td>
<td>NS</td>
</tr>
<tr>
<td>Hypothermia</td>
<td>5 (20.8)</td>
<td>-</td>
<td>HS</td>
</tr>
<tr>
<td>Oliguria/Anuria</td>
<td>4 (16.7)</td>
<td>-</td>
<td>HS</td>
</tr>
<tr>
<td>Diarrhea/vomiting/abdominal distension</td>
<td>6 (25)</td>
<td>1 (7.1)</td>
<td>HS</td>
</tr>
<tr>
<td>Cough/Nasal discharge</td>
<td>3 (12.5)</td>
<td>2 (14.3)</td>
<td>NS</td>
</tr>
<tr>
<td>Tachypnea/grunt/chest indrawing</td>
<td>5 (20.8)</td>
<td>1 (7.1)</td>
<td>HS</td>
</tr>
<tr>
<td>Intermittent apnea/cyanosis</td>
<td>4 (16.7)</td>
<td>-</td>
<td>HS</td>
</tr>
<tr>
<td>Umbilical or skin sepsis</td>
<td>5 (20.8)</td>
<td>2 (14.3)</td>
<td>NS</td>
</tr>
<tr>
<td>Hemorrhage/Seizures</td>
<td>4 (16.7)</td>
<td>-</td>
<td>HS</td>
</tr>
</tbody>
</table>

HS: highly significant  NS: non significant  S: significant
* Vaginal discharge, skin infection, burning in urine, and/or fever during 7 days before delivery.
** Premature rupture of memberanes, prolonged labor.
*** Foul smelling/purulent vaginal discharge, fever, diarrhea, persistent cough.
Table 2. Laboratory investigations for 24 septic neonates versus 14 control neonates

<table>
<thead>
<tr>
<th>Investigation</th>
<th>Septic neonates n=24</th>
<th>Control neonates n=14</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total WBCs ( / cc)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X±SD</td>
<td>10920 ± 5301</td>
<td>9883 ± 3176</td>
<td>NS</td>
</tr>
<tr>
<td>Range</td>
<td>3700 – 18000</td>
<td>8010-12630</td>
<td></td>
</tr>
<tr>
<td><strong>Percent of Band cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X±SD</td>
<td>7.17 ± 3.6</td>
<td>7.1 ± 3.2</td>
<td>NS</td>
</tr>
<tr>
<td>Range</td>
<td>3-10</td>
<td>5-10</td>
<td></td>
</tr>
<tr>
<td><strong>C-reactive protein (mg/L)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X±SD</td>
<td>56.7 ± 42.2</td>
<td>6.3 ± 1.2</td>
<td>HS</td>
</tr>
<tr>
<td>Range</td>
<td>12 – 96</td>
<td>0-9</td>
<td></td>
</tr>
<tr>
<td><strong>Interleukin-18 (pg/ml)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X±SD</td>
<td>1429.1 ±801</td>
<td>129.7±120</td>
<td>VHS</td>
</tr>
<tr>
<td>Range</td>
<td>110-2991</td>
<td>0 – 183</td>
<td></td>
</tr>
</tbody>
</table>

NS: non significant  HS: highly significant  VHS: Very highly significant

**Figure (2)** illustrates levels of circulating interleukin-18 (cIL-18) in 24 septic neonates (O) and 14 control neonates (▲) on ROC curve, cut-off point : 188 pg/ml. The reproduced sensitivity, in neonates with positive blood cultures, accounted for 91.7% and the reproduced specificity, in control neonates accounted for 85.7%.

**Figure (3)** illustrates serum levels of C-reactive protein (CRP) in 24 septic neonates (O) and 14 control neonates (▲) on ROC curve, cut-off point 10 mg/L. The reproduced sensitivity in septic neonates with positive blood culture, accounted for 72 %, and the reproduced specificity, in control neonates, accounted for 100%.

**Figure (3)** shows that the two septic neonates with cIL-18 levels below 188 pg/ml (false negatives) have serum CRP levels, on ROC curve, of 96 mg/L and 24 mg/L. This raises the sensitivity of combined cIL-18 and serum CRP for the diagnosis of neonatal sepsis into 100%.
Figure 2: Scattergram of serum IL-18 levels of 24 septic neonates (with positive blood cultures) and 14 control neonates.
**DISCUSSION**

The early and efficient diagnosis of neonatal sepsis remains a difficult task, as the first clinical symptoms and signs are non-specific. If treatment is delayed until symptoms and signs become obvious, the risk of preventable mortality would be brought up (16).

Blood culture is widely used for the diagnosis of septicemia. However, in many clinical situations the yield from blood culture is low, positive cultures are obtained from fewer than 30%. Some of the patients with false negative blood culture may have had prior antibiotic treatment or they were not bacteremic at the time of blood collection (9). In this study, positive blood cultures were obtained from 40% of neonates clinically suspected with sepsis. This quite higher rate may be due to the fact that neonates who have received antibiotic therapy were excluded.

The significant prevalence of E. coli sepsis observed in this study was reported by other studies (3,9,17,18). Although the use of IAP has been effective in reducing GBS infections it resulted in an increased incidence of infections caused by pathogens other than GBS, including anti-microbial resistant strains and E. coli (18).

Among 24 septic neonates, significantly associated maternal events included vaginal discharge, skin infection, burning in urine (in the last trimester) and/or fever during 7 days before delivery, PROM and/or prolonged labor. Meanwhile, clinical neonatal criteria significantly associated with sepsis included un-consciousness, week cry, sucking weak or stopped (for >8h), limbs become limp, hypothermia, oliguria/anuria, diarrhea/vomiting/abdominal distension, tachypnea/grunt/chest indrawing, intermittent apnea/cyanosis, and hemorrhage/seizure. Meanwhile, fever, cough/nasal discharge, and umbilical/skin sepsis were not significant associates. Similar results were obtained by Bang and Co-Workers (7).

There is no single diagnostic test that can reliably diagnose sepsis in the newborn. The proof of sepsis depends primarily on...
recovery of the infecting organisms from blood (19).

Of the laboratory tests, carried out for culture-proven septic neonates, only serum CRP and cIL-18 levels showed significant rise compared with that of control neonates. Other parameters proved to be non-significant associates of sepsis.

There is not an established standard of practice for the use of CRP in infants, and a variety of approaches are described. Some authors advocate the use of serial CRP levels as an early diagnostic tool for confirming the presence of sepsis, whereas others view it as a screening tool to rule out the presence of sepsis. Further, some authors advocate the use of CRP to monitor antibiotic therapy. Others do not use it at all (2). However, ROC curve analysis demonstrated substantially high mean specificity, consuming cut-off point values of 1-77 mg/L for septic and 0.1-6 mg/L for healthy neonates (3).

The catalog of proteic molecules associated with sepsis is extensive and includes cytokines, chemokines, adhesion mediators, soluble receptors, and acute-phase proteins (20). The standard method for characterizing diagnostic utility is the ROC curve, which plots the sensitivity (true-positive diagnoses) of the diagnostic marker(s), at a specified cut-off value, against the specificity (false-positive diagnoses) (1). In this study, we found that the sensitivity of cIL-18 and serum CRP is 91.7% and 72%, and the specificity is 85.7% and 100%, respectively. When both cIL-18 and serum CRP combined are used, both the sensitivity and specificity rose to 100%.

Ideally, a clinical laboratory test that is considered reliable and diagnostic must have a high level of sensitivity and specificity in identifying what is measuring. A test used to diagnose sepsis neonatorum must always indicate abnormal results in those infants who have sepsis (sensitivity) and indicate normal results in infants who are not infected (specificity). Because neonatal sepsis has a reported mortality rate between 5% and 50%, it is critical that practitioners identify all infants with sepsis (high sensitivity) (2).

The sensitivity of CRP in the diagnosis of neonatal sepsis is quite low. However, when combined with other tests, elevated CRP levels support a diagnosis of sepsis while awaiting culture results (3).

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11. Dean AG, Dean JA, Coulombier D, Brendel KA. Epi-Info (version 6.1): A word processing, database and statistics program for epidemiology and micro-
computer office. CDC 2000. Atlanta, Georgia, USA.


**Study**

**Objective:**

To evaluate the effectiveness of using the 

**Methods:**

- **Study Population:**
  - Children with gastrointestinal symptoms
  - Controls: healthy children

**Results:**

1. **Detection Rate:**
   - 41% of children with symptoms were positive for
   - 26% of children without symptoms were positive for

2. **Sensitivity:**
   - 19.7% of children with symptoms were detected
   - 27% of children without symptoms were detected

**Conclusion:**

The use of the test in children with gastrointestinal symptoms is recommended, as it showed a significant increase in detection rate. However, the test's sensitivity in healthy children is not ideal. Further studies are needed to optimize the test's use.