Assessment of Interleukin-27 and Chemokine RANTES as Biomarkers for Early Onset Neonatal Sepsis

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Early-onset neonatal sepsis (EONS) is a global health problem with high morbidity and mortality rates. Early diagnosis is a critical issue in determining treatment strategies. There is no single diagnostic test that can fulfill all requirements of the ideal biomarker yet. The current study enrolled 47 cases with EONS, admitted to the Neonatal Intensive Care Units at Beni-Suef University teaching Hospital from February 2017 to November 2017 and 37 apparently healthy controls. All were subjected to routine laboratory tests and serum concentration of IL-27 and regulation on activation normal T-cell expressed and secreted (RANTES) were measured. Significantly higher concentrations of IL-27 were observed in the septic group while RANTES were significantly lower in comparison to the controls. Moreover, there were no significant correlations between levels of IL-27 and RANTES either in the septic or the control group. Sensitivity, specificity, positive and negative predictive values for IL-27 were 93.6%, 81.1%, 86.3% and 90.9, respectively while for RANTES such values were 68.1%, 78.4%, 80% and 65.9%, respectively. A combination of both markers showed 97.3% specificity for sepsis. In conclusion, IL-27 is a useful and sensitive biomarker either individually or combined with other candidate biomarkers like RANTES.

Neonatal sepsis is a bacterial infection of the blood that occurs during the first 28 days of life. It is a global health problem represents one of the main causes of morbidity and mortality of newborns notably in developing countries. Depending upon the onset of the clinical signs, neonatal sepsis is classified into an early onset and a late onset sepsis. Early onset neonatal sepsis (EONS) is defined as sepsis that manifest during the first 72 hours of life [1, 2].

Rapid and precise diagnosis of sepsis is an essential tool to the effectiveness of treatment and proper outcomes [3, 4]. Unfortunately, clinical diagnosis of EONS is remarkably confusing due to the nonspecific and crossover manifestations that may be presented in various non-infectious diseases [5]. Although blood culture technique is the most reliable test for diagnosis of neonatal sepsis, it is time consuming with false positive and false negative results which represents a significant challenge for the life of newborn infants. Other laboratory tests including complete blood cell count, differential, immature-to-total neutrophils (I/T) ratio, alongside with C-reactive protein (CRP) are equally helpful markers in combination with blood culture, but still insufficient and non-specific. A single and specific test that can provides an early and reliable diagnosis of neonatal sepsis is unavailable yet. Therefore, it is essential to
identify new biomarkers that meet these features [6, 7].

The pathogenesis of sepsis is relatively complex, but in apart, the immune system response is an outstanding player in its propagation by deregulating innate immune system components including macrophages, monocytes and the released cytokines [8-10]. During septicemia different clusters of inflammatory cytokines are produced, their over-activity may cause tissue damage and promote pathogenesis [11]. While experimental and clinical studies have revealed conflicting roles of cytokines during inflammation, we need to understand the signaling pathway of cytokines involved in pathogenesis of sepsis that allow optimized diagnostic and therapeutic approaches [12, 13].

Interleukin 27 (IL-27) is a pleiotropic heterodimeric cytokine belonging to the IL6 and IL-12 families of cytokines. It is produced by activated macrophages, endothelial cells, and dendritic cells. It binds to the cytokine receptor complex composed of IL-27 R alpha/WSX-1/TCCR and gp130 [14]. Paradoxical effects of IL-27 as pro-inflammatory and anti-inflammatory agent, has a wide range of immune responses from regulation of CD4 cell development, suppression of T-cell proliferation, stimulate activity of CD8 cell, and induce class switching of B-cells, which has diverse effects on cells of the innate immune system [15, 16]. IL-27 potentiate the early phase of TH1 response and suppress TH2 and TH17 differentiation through induction of IL-12 receptors on naïve TH cells making them susceptible to subsequent IL-12 activity [17].

Chemokine ligand 5 (CCL5) also known as RANTES (regulation on activation normal T-cell expressed and secreted) is a C-C motif chemokine. It has been identified as one of the essential HIV suppressive factor produced by activated CD8+ T cells. It is also produced by many cells of the body including macrophages, platelets, eosinophils, and fibroblasts, epithelial and endothelial cells [18-20]. It signals through several receptors including CCR1, CCR3, CCR5 and US28. CCL5 is a potent chemoattractant for effector memory T cells (CD4+/ CD45RO+), B cells, monocytes, mast cells, basophiles, eosinophils, immature dendritic cells and natural killer cells [21].

We aimed to assess the usefulness of the biochemical markers, namely, interleukin-27 (IL-27) and RANTES, both individually and in combination for diagnosis of early onset neonatal sepsis.

Materials and Methods

Inclusion criteria and group allocation

This is a case-control study that was carried out at the neonatal intensive-care unit (NICU) of Beni-Suef University Hospital over a period of ten months. The study included 84 neonates admitted to NICU; 47 cases diagnosed as early onset neonatal sepsis and 37 apparently healthy neonates as a control group. The study protocol was reviewed and approved by the ethical committee of the Faculty of Medicine, Beni-Suef University (FMBSU REC/07072019). Informed consent was obtained from parents of all neonates enrolled in this study.

All neonates suspected to have sepsis were subjected to a septic screen and bacterial blood cultures, performed to corroborate the diagnosis. Clinical and historical suspicion of neonatal sepsis included the presence of two or more of the following features (1) Apnea, Tachypnea, Respiratory Distress, Cyanosis (2) Bradycardia, Tachycardia (3) Hypotonia, Seizures (4) poor skin color with poor perfusion (5) Irritability, lethargy, poor feeding (6) Hepatomegaly, splenomegaly, abdominal distention (7) Hyperthermia, hypothermia (8) Antenatal risk factors: mothers Inadequately treated against Group B Streptococcus (GBS) colonization, unknown GBS status with premature rupture of membranes,
maternal chorioamnionitis, preterm labor, twins gestation, fetal distress and maternal temperature.

The various components of the septic screen included total leucocytic count, leucocytosis (WBC count > 35000/mm³), leucopenia (WBC count < 5000/mm³), thrombocytopenia (100 × 10³/mm³), anemia (hemoglobin concentration < 14.5 g/dL) and C-reactive protein (CRP>6 mg/dL). Full maternal history was taken, and a detailed perinatal history with a thorough clinical examination was conducted for all neonates enrolled in the study.

Full maternal history was taken, and a detailed perinatal history with a thorough clinical examination was conducted for all neonates enrolled in the study. Newborn infants with congenital infection, suspected inborn error of metabolism, perinatal asphyxia, congenital anomalies, chromosomal abnormalities or negative CRP were excluded from the case group.

Criteria must be fulfilled for inclusion to the control group were neonates with neither clinical feature of infection nor perinatal risk factors with negative sepsis markers and negative blood culture.

Biochemical and Microbiological laboratory tests

A Complete blood count (CBC) was typically done on automated cell counter, Cell Dyn 1800 (Abbott diagnostics, Germany), C-reactive protein (CRP) was done using latex agglutination test (Omea diagnostics LTD, UK). Blood cultures were obtained as routine in all cases and performed using BACTEC FX 40 automated blood culture system (Becton Dickinson, Heidelberg, Germany) according to the manufacturer instructions.

Measurement of serum level of IL-27 and RANTES

A sample of 1 ml venous blood was withdrawn into a test tube without anticoagulants. The sample was allowed to clot for 30 minutes and then centrifuged at 1000 × g for 10 minutes at 4°C. Each serum sample was divided into 3 equal aliquots and stored at -20°C until further analysis. Serum levels of IL-27 and RANTES were assessed using solid phase sandwich ELISA Picokine ELISA kit for II-27 (Catalogue number: EK0799) and QuantiKine. R&D systems for RANTES (Catalogue number: DRN00B). The proportional color changes in the samples were measured using Stat fax 2100 microplate reader (Awareness Technology, Inc., USA).

Statistical Analysis

Statistical analysis was conducted using SPSS computer program (version 20 windows.). Results are expressed as mean ± standard deviation or number (%). Comparison between categorical data [Number (%)] was done using Fisher exact test or Chi square test if cell count was less than five. Test of normality, Shapiro-Wilk test, was used to measure the distribution of data. Accordingly, comparison between variables in the two groups was done using either unpaired t test or Mann Whitney test whenever it was appropriate. Correlation between levels of IL-27 and RANTES in both groups was done using Spearman’s Rank correlation coefficient. The receiver operating curve (ROC) test was used to determine the diagnostic indices (specificity, sensitivity, predictive values and accuracy) of both IL-27 and RANTES. P value of ≤ 0.05 was considered significant.

Results

A total of 84 neonates was enrolled in this study and classified into two groups. Early onset neonatal sepsis group (EONS) involved 47 neonates (29 males and 18 females) with a mean age (27.04 ± 3.3 hour) and a control group that involved 37 neonates (22 males and 15 females) with a mean age (33.44 ± 4.0 hour). The mean of gestational age in weeks for the sepsis group was (35.66 ± 2.63) and for the control group was (36.30 ± 1.93). The mean of neonatal weight for the sepsis group was (2.95 ± 0.84 kg), while that of controls was (2.79 ± 0.69 kg) as in Table 1.

IL-27 and RANTES were measured in 47 septic neonates and 37 controls. According to frequency of clinical manifestations; neonates included in the sepsis group were presented with respiratory distress, jaundice, convulsions, hypoglycemia, cyanosis and hypothermia respectively.
Table 1. Demographic features and hematological parameters of EONS and control group and their P values.

<table>
<thead>
<tr>
<th></th>
<th>EONS (n= 47)</th>
<th>Control (n= 37)</th>
<th>P-value</th>
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<tbody>
<tr>
<td>Age (hrs. ± Std.Deviation)</td>
<td>26.4 ± 15</td>
<td>34.44 ± 20</td>
<td>NS</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>18 (38.3%)</td>
<td>15 (40.5%)</td>
<td>NS</td>
</tr>
<tr>
<td>Male</td>
<td>29 (61.7%)</td>
<td>22 (59.5%)</td>
<td></td>
</tr>
<tr>
<td>Gestational age (wks.)</td>
<td>35.66 ± 2.63</td>
<td>36.30 ± 1.93</td>
<td>NS</td>
</tr>
<tr>
<td>Vaginal delivery</td>
<td>7 (18.9%)</td>
<td>4 (8.5%)</td>
<td>NS</td>
</tr>
<tr>
<td>Stay in NICU (day)</td>
<td>10.73 ± 6.12</td>
<td>..................</td>
<td>..........</td>
</tr>
<tr>
<td>Birth weight (kg.)</td>
<td>2.79 ± 0.69</td>
<td>2.95 ± 0.84</td>
<td>NS</td>
</tr>
<tr>
<td>Hb (gm/dl)</td>
<td>11.957± 2.9</td>
<td>17 ± 1.2</td>
<td>0.001</td>
</tr>
<tr>
<td>TLC (×10^3/mm^3)</td>
<td>16.75 ± 4.89</td>
<td>12.01± 1.98</td>
<td>0.001</td>
</tr>
<tr>
<td>ANC/mm^3</td>
<td>1263±640</td>
<td>8500±27·</td>
<td>0.001</td>
</tr>
<tr>
<td>I/T ratio</td>
<td>0.42±0.11</td>
<td>0.11 ± 0.04</td>
<td>0.001</td>
</tr>
<tr>
<td>Platelets (×10^3/mm^3)</td>
<td>221.68± 68.23</td>
<td>225 ± 51.76</td>
<td>NS</td>
</tr>
</tbody>
</table>

*P > 0.05 is not significant (NS)*

**Measurements of IL27 and RANTES**

Interleukin-27 was highly expressed in the serum of septic neonates (545 ± 210 pg/ml) compared to the control group (212 ± 186 pg/ml); P= 0.001 (Figure 1). The level of RANTES was significantly lower in the EONS group (473 ± 310 pg/ml) in comparison to the level in the control group (333 ± 337 pg/ml); P= 0.052. There was no significant correlation between levels of IL-27 and RANTES in both the neonatal sepsis groups (R^2= 0.02; P= 0.2) and the control (R^2= 0.048; P= 0.059).

ROC curve analysis for each biomarker was separately analyzed (Figure 2). All diagnostic indices of IL-27 were high when compared with those of RANTES, except a slight decrease in specificity of IL-27 (Table 2). Assessment of usefulness of combination of IL-27 and RANTES revealed 97.3 % specificity and 96.8 % PPV but showed low sensitivity and NPV.
Figure 1. Scattered plot of serum IL-27 (A) and RNATES (B) expressed in pg/ml in the two studied groups.
Figure 2. ROC curve analysis of serum IL-27 (A) and RANTES (B).

Table 2. Diagnostic indices of IL-27 and RANTES individually and in combination.

<table>
<thead>
<tr>
<th></th>
<th>Cut-off</th>
<th>AUC (95% CI)</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
<th>Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-27</td>
<td>&gt; 316</td>
<td>0.873 (0.78 - 0.96)</td>
<td>93.6%</td>
<td>81.1%</td>
<td>86.3%</td>
<td>90.9%</td>
<td>88.1%</td>
</tr>
<tr>
<td>RANTES</td>
<td>&gt; 366</td>
<td>0.666 (0.54 - 0.79)</td>
<td>68.1%</td>
<td>78.4%</td>
<td>80.0%</td>
<td>65.9%</td>
<td>72.6%</td>
</tr>
<tr>
<td>IL-27/ RANTES</td>
<td>------</td>
<td>-----</td>
<td>63.8%</td>
<td>97.3%</td>
<td>96.8%</td>
<td>67.9%</td>
<td>78.6%</td>
</tr>
<tr>
<td>(Combined)</td>
<td></td>
<td></td>
<td>63.8%</td>
<td>97.3%</td>
<td>96.8%</td>
<td>67.9%</td>
<td>78.6%</td>
</tr>
</tbody>
</table>
Several risk factors were observed at different rates in the neonatal sepsis group. These include, premature rupture of membrane (PROM)>12 hours (20.45%), followed by preterm (18.18%), chorioamnionitis (9.09%), maternal fever (6.82%), fetal distress (4.55%) and finally twin gestation (4.55%). No risk factors were detected in 36.36% of septic neonates.

Mean values of hemoglobin and absolute neutrophil count (ANC) were significantly decreased in septic patients when compared to controls (P= 0.001). The mean values of both TLC and I/T ratio were significantly higher in septic patients when compared with their values in the control group (P= 0.001). However, there was no significant difference in the mean value of platelets in both groups (P= 0.812; Table1).

Blood culture test of the EONS group revealed *Klebsiella pneumoniae* as the major detected organisms followed by methicillin resistant *Staphylococcus aureus* (MRSA); while *Escherichia coli* (*E. coli*) and coagulase negative *Staphylococcus* (CoNS) were detected in equal numbers, and both *Candida* and *Enterococcus* was the minor detected organisms (Figure 3).

Figure 3. Result of blood culture in neonatal sepsis group.

Discussion

Rapid and precise diagnosis of neonatal sepsis is a pivotal process, as it augments the effectiveness of medical treatment leading to a proper outcome. An accurate ruling out of sepsis is also critical as it allows avoidance of neonatal exposure to broad-spectrum antibiotics and reduces the risk of emergence of antibiotic resistant bacterial strains [22].

Although Lancefield group B streptococcus (*Streptococcus agalactiae*) represent the major pathogen responsible for early onset sepsis [23]. The current study showed *Klebsiella pneumoniae* was the most frequent isolated organism from blood culture in neonatal sepsis group, this difference may be attributed to intrapartum administration of ampicillin. Our result is in agreement with other previous studies reported the same finding [24-26].

An ideal biomarker for diagnosis of neonatal sepsis must have high sensitivity, specificity and predictive values. It is critical to be obtained easily. The change in its level
should occur in the earlier stage of sepsis and stabilized for a sufficient period of time to give the chance for its measurement and guides management. Moreover, the level of this biomarker is directly proportional to severity of disease. In addition, allow prediction of the prognosis and finally can differentiate between causes of sepsis including bacterial, fungal and viral causes [27].

Results of IL-27 in the current study are comparable to other studies conducted on septic patients at different age including neonates, children and adults. Values of IL-27, reported by other studies, were more than one fold higher in septic neonates compared to control groups [28-30]. An earlier study was conducted on infected and non-infected EONS sepsis neonates to evaluate the diagnostic value of multiple biomarkers including IL-27 using magnetic bead-based multiplex assay [31]. Their data showed IL27 as a potential marker for preliminary diagnosis of sepsis. Our data revealed better diagnostic precision, based on predictive values, for IL27 when compared to these of He, et al., (2017). These differences in predictive values may be attributed to the method of IL-27 assay, cutoff level, and sample size. Another possible explanation is their difference in study group allocation, add to this except cord blood samples, they did not get blood samples from non-septic neonates.

The current study demonstrated significantly reduced levels of RANTES in the neonatal septic group compared to control neonates. These data are in agreement with previous studies, reported the similar findings [32-34]. Our results were contradicted with the investigation of Stojewska et al., (2016) who reported that levels of RANTES were higher in septic neonates compared to non-infected ones [35]. Another study stated that levels of the chemokine RANTES were low in EONS group compared to the non-infected neonates, however the difference was not statistically significant [36]. This inconsistency in findings between different studies may be due the differences in sample size, onset of sepsis and study design. Shouman & Badr (2009) conducted a study on relatively small sample size of 15 septic neonates and 15 controls for assessment of RANTES and tumor necrosis factor-alpha using ELISA technique. Their study included neonates with both early and late onset sepsis. ROC curve analysis of their results revealed a lower sensitivity and specificity for RANTES compared to these observed in the current study. These differences in diagnostic parameters may be due to their relatively small sample and to the differences in time of onset of illness between the two studies. Although most diagnostic parameters of RANTES in the present study were lower than IL-27, a combined measurement of both biomarkers revealed a 97.3% specificity and 96.8% PPV.

In the present study there was practical limitation due to ethical reasons. We were incompetent to withdraw more than one sample to perform a serial measurement of IL-27 and RANTES to assess dynamic changes in their levels in relation to changes in severity of disease.

In conclusion, IL-27 is a promising biomarker for diagnosis of neonatal sepsis. Further studies with a considerable number of cases are needed for evaluation of IL-27 either individually or in combination with RANTES or any other potential biomarkers. In septic neonates a serial measurement of cytokines levels is also required to assess their validation in prediction of disease prognosis and response to treatment.
Acknowledgments

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