

Neutrophil CD11b, CD64 and Lipocalin-2: Early Diagnostic Markers of Neonatal Sepsis

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Neonatal sepsis remains a global health problem particularly in the developing countries. Its diagnosis remains one of the most difficult issues in clinical medicine. Many immunologic markers including neutrophils CD11b and CD 64 and Lipocalin-2 have been tested as biomarkers of neonatal sepsis. The aim of the present study was to assess the value of these markers for early diagnosis of neonatal sepsis. The study included 60 neonates with suspected neonatal sepsis and 20 apparently healthy controls. Lipocalin-2 serum level was assessed by ELISA while neutrophils CD11b and CD64 expressions were evaluated by flow cytometry. Neutrophils CD64 and CD11b expression levels elevated significantly in cases (67.8 ± 7.57) and (57.01 ± 2.46) respectively than in controls (11.78 ± 7.20) and (8.26 ± 4.79). Lipocalin-2 serum level was significantly higher in the patients (145.3 ± 55.3) than in controls (22.4 ± 12.9). In conclusion, neutrophils CD64, CD11b and Lipocalin-2 are early, specific and sensitive diagnostic markers of neonatal sepsis.

Neonatal sepsis is a life-threatening condition which constitutes a major cause of neonatal morbidity and mortality [1]. The definitive diagnosis of neonatal sepsis, particularly at its early stage is difficult and remains a challenge for the health care providers. Blood culture is considered the current gold standard technique for the clue diagnosis of bacterial neonatal sepsis [2]. Despite its established merit in differentiating sepsis from non-infectious conditions, the blood culture lacks the sensitivity and specificity, and often there is a substantial time delay [3]. Herein, the introduction of a certain marker (s) would not only provide a reliable alternative to blood culture but also give the opportunity for early treatment of neonatal sepsis, thereby decreasing its sequels dramatically.

Many biomarkers including white blood cells count; acute phase reactants such as C-reactive protein (CRP), procalcitonin and serum amyloid alpha; cytokines and other

protein markers such as tumor necrosis factor- α , interleukin-6, interleukin-8 and complement protein C3a have been used extensively for diagnosis of neonatal sepsis [4].

C-reactive protein (CRP) is an acute-phase reactant produced by hepatocytes and normally present at very low level. Following tissue damage or infection, its serum level elevated within few hours and peaked at around 48 hours [5]. Although, CRP is one of the most widely available and most used laboratory tests for neonatal sepsis, it lacks diagnostic accuracy and sensitivity [6].

Lipocalin-2, also known as neutrophil gelatinase-associated lipocalin (NGAL) or siderocalin was originally identified as a component of neutrophil granules and regarded as a specific marker of neutrophil activity [7]. Since then, its expression has been observed in most tissues, and its synthesis was induced in the epithelial cells during inflammation [8]. The major

biological role of lipocalin-2 is its bacteriostatic feature by binding to the bacterial siderophores and limiting bacterial iron supply [9]. Moreover, it has the advantage to discriminate between viral and bacterial infections [10].

Recent advances in flow cytometric technology have paved the way for detection of numerous cell surface antigens such as CD11b and CD64 which could be used as potential promising biomarkers of infection [11]. CD64 is a high affinity Fc γ receptor I (Fc γ RI) which is expressed constitutively on the monocytes, and to a very low extent on the resting polymorphonuclear cells (PMNs) [12]. Upon antigenic stimulation by the bacterial products, the CD64 expression on PMNs increases remarkably in an attempt by the innate immune response to increase phagocytosis. Thereafter it returns to its basal level in a few days following fading the stimulus away [11]. These favorable properties could make the neutrophil CD64 a potential useful indicator for detecting the early stage of immune response to bacterial infection and consequently as a diagnostic marker for neonatal sepsis.

CD11b/CD18 is an α -subunit of the β_2 -integrin adhesion molecule which under normal circumstances is expressed at a very low concentration on the surface of non-activated neutrophils [13]. This expression increases exponentially within a few minutes after activation of the neutrophils by the bacteria and/or its endotoxins [14]. This unique property could render CD11b a potential early marker for detection of bacterial infection.

It is well known that the early diagnosis of neonatal sepsis is not only challenging but its reliable diagnosis at the time of clinical suspicion might be also impossible. Thereby, the aim of the current study is to evaluate the value of peripheral blood

neutrophils CD11b and CD64 expression levels and serum lipocalin-2 as early diagnostic markers of neonatal sepsis.

Patients and Methods

Study Design and Patients

This is a case-control study which was conducted during the period from April 2016 to September 2016 at the Central Research Laboratory in collaboration with the Neonatal Intensive Care Unit (NICU), Sohag Faculty of Medicine, Egypt. A total of 60 neonates (study group) who were admitted to the NICU with manifestations suggestive of sepsis or who developed signs of sepsis while in the ward were enrolled into the study. Neonatal sepsis was suspected according to the international criteria for bacterial sepsis [15, 16]. The exclusion criteria were, prematurity (< 37 weeks), low birth weight (< 2500 g), congenital anomalies, neonates to mothers with gestational diabetes and neonates on current antibiotic therapy. A total of 20 healthy full term neonates without any manifestations of sepsis, born after uncomplicated pregnancy and delivery were enrolled as a control group. The study protocol was approved by the Ethics committee of Sohag Faculty of Medicine, Sohag University and written informed consents were obtained from all parents.

Detailed medical history was obtained from the parents of the newborns followed by thorough clinical examination of the enrolled neonates. Under complete aseptic conditions, blood samples were withdrawn from all participants. The neonates of the study group were screened for sepsis with the following laboratory tests; blood cultures, complete blood count (CBC) and differential leucocytic count. All neonates in both the study and control groups were investigated for neutrophil surface markers (CD11b and CD64) expression, lipocalin-2 and CRP serum levels.

Blood Culture

Blood culture bottles (Wampole isolator 1.5; Oxoid Ltd, UK) were incubated at 37°C for at least 7 days under aerobic conditions. Subcultures were done every 48 hours. The growing colonies underwent further morphological and biochemical identification to identify the different bacterial species according to the standard microbiological methods [17].

CRP Assay

Serum CRP level measured semi-quantitatively by latex agglutination technique (Omega Diagnostics kits, UK) according to the manufacturer's instructions. It was considered positive when the titer was ≥ 6 mg/L.

Serum Lipocalin-2 assay

Serum Lipocalin-2 was assayed using Quantikine ELISA Kit (R&D Systems, Minneapolis, USA) according to the manufacturer's protocol. This assay employs the quantitative sandwich enzyme immunoassay technique, and results were read using the Stat fax 2600 microplate reader (Awareness Technologies, Palm City, USA).

Flowcytometric analysis of peripheral blood neutrophils CD11b and CD64 levels

An aliquot of 100 μ l of anti-coagulated blood from each study participant was stained with 10 μ l Phycoerythrin (PE) conjugated monoclonal antibodies namely anti CD11b-PE and anti CD64-PE (Beckman Coulter, France). Tubes were vortexed then incubated for 15 min at room temperature (RT) in the dark. One ml of lysing solution (Versalyse TM; Beckman Coulter, France) was added to the mixture while vortexing followed by incubation for 15 min at RT in the dark. Finally, analysis of the cells was done by EPICS XL flow cytometry using SYSTEM II version 3.0 software (Beckman Coulter, USA) with a standard 4-color filter configuration. The neutrophils were specifically analyzed by selective gating based on the parameters of forward (size) and side (granularity) scatter. In each sample, 10,000 cells were analyzed. Gates were analyzed for number and percentage of cells.

Statistical Analysis

Statistical analysis was carried out using SPSS (statistical program for social science version 12). Data were expressed as mean $\pm$ SD or number and percent. The student t-test and the Mann-Whitney U test were used to compare the normally and unevenly distributed data respectively. P value of <0.05 was

considered significant for all statistical tests. The sensitivity, specificity, positive (PPV) and negative predictive values (NPV) of CRP, CD11b, CD64 and serum lipocalin-2 for diagnosis of neonatal sepsis were calculated considering the blood culture as the gold standard reference test.

Result

A total of 60 neonates with suspected neonatal sepsis (study group) and 20 without evidence of sepsis (control group) were recruited for the study. The characteristics, the clinical presentations and the laboratory findings of the participants are displayed in table 1.

The blood culture results revealed that 39 out of 60 neonates (65%) with suspected sepsis were positive (proven sepsis). The most commonly isolated organisms were; *Klebsiella Pneumonia* (n=12, 30.7%), *Staphylococcus aureus* (n=9, 23.1%), *Escherichia coli* (n=8, 20.5%), Coagulase negative *Staphylococcus* (n=5, 12.8%), *Streptococci agalacti* (n=4, 10.3%) and *Enterobacter aerogens* (n=1, 2.6%) (Data are not shown in the table).

The laboratory investigations showed that, CRP and lipocalin-2 serum levels were significantly higher in cases (36.1 ± 31.7) and (145.3 ± 55.3), respectively than in controls (5.3 ± 12.0) and (22.4 ± 12.9), respectively ($P < 0.001$) (table 2). There was a significant increase in the neutrophils CD11b and CD64 expression levels in cases (57.1 ± 2.5) and (67.8 ± 7.6), respectively than their levels in the controls (11.8 ± 7.2) and (8.3 ± 4.8), respectively ($P < 0.001$) (Table 2).

Table 1. The participants' characteristics.

Characteristic	Patients (n=60)	Controls (n=20)	P-value
Age (days)	9.9±6.7	8.3±4.5	NS
Gender			
Male	34 (56.7%)	12 (60%)	< 0.05
Female	26 (43.3%)	8 (40%)	
Weight (Kg)	2.7±0.7	2.9±0.5	NS
Clinical manifestations			
a. General	43 (71.7%)		
b. Respiratory	38 (63.3%)		
c. Cardiovascular	22 (36.7%)	_____	
d. Neurologic	48 (80.0%)		
e. Gastrointestinal	45 (75.0%)		
Laboratory findings			
a- Complete blood count			
Leucocytosis ($> 20 \times 10^9/L$)	26 (43.3%)		
Leucopenia ($< 5 \times 10^9/L$)	13 (21.7%)		
I/T ratio (> 0.2)	38 (63.3%)	_____	
Thrombocytopenia ($< 150 \times 10^9/L$)	22 (36.7%)		
b- Blood culture			
Negative culture	21 (35.0%)	_____	
Positive culture	39 (65.0%)		

Variables are expressed as Mean $\pm$ Standard deviation, or number (percentage), $P>0.05$ is not significant (NS).
Abbreviation: I/T ratio: immature/total neutrophil ratio

Table 2. Levels of the studied markers (neutrophil CD11b, CD64, lipocalin-2 and CRP) among cases and controls.

Variable	Cases (n=60)	Controls (n=20)	P-value
CD11b	57.1±2.5	11. 8±7.2	< 0.001
CD64	67.8±7.6	8.3±4.8	< 0.001
Lipocalin-2 (ng/ml)	145.3 $\pm$ 55.3	22.4 $\pm$ 12.9	< 0.001
CRP (mg/L)	36.1 $\pm$ 31.7	5.3±12.0	< 0.001

Variables are expressed as Mean $\pm$ Standard deviation, CRP: C-reactive protein
 $P<0.05$ is significant.

The neutrophil CD11b, CD64 and lipocalin-2 showed higher diagnostic accuracy than CRP. The CD64 has a higher sensitivity and specificity than CD11b for diagnosing neonatal sepsis. Although, Lipocalin-2 has

high sensitivity and specificity comparable to CD11b and CD64; it has the lowest negative predictive value [table (3) and figure (1)].

Table 3. Comparison of the sensitivity, specificity, PPV and NPV of the studied markers (CD11b, CD64, Lipocalin-2 and CRP).

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
CD11b	95	95	94.1	95.9
CD 64	97	100	97.4	100
Lipocalin-2	90	95	97.2	82.6
CRP	73	85.4	70.3	88

PPV: positive predictive value, NPV: negative predictive value.

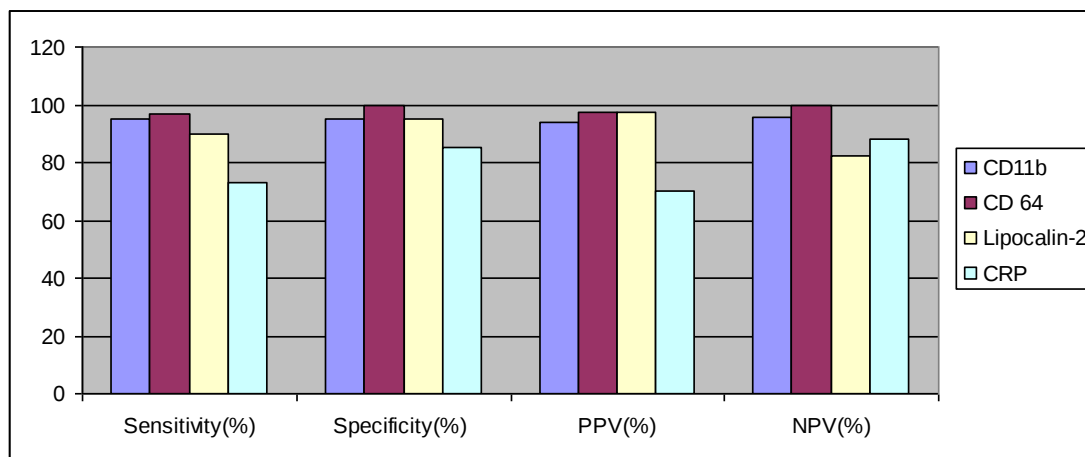


Figure 1. Performance of the studied diagnostic markers of neonatal sepsis.

Discussion

The present study reported a significantly high level of neutrophils CD64 in the presence of sepsis. This marker showed the highest sensitivity (97.4% and specificity (100%) for diagnosis of neonatal sepsis. The reported sensitivity and specificity of CD64 coincides with the figures reported by previous studies (18- 20). Moreover, Ng and his college reported that amongst four cell

surface markers; neutrophil CD11b and CD64, and lymphocyte CD25 and CD45RO, CD64 had the highest sensitivity (95–97%) and specificity (88–90%) at the onset and up to 24 h after the initial clinical presentation for diagnosing late-onset bacterial infection and necrotizing enterocolitis [14]. Multiple previous studies showed nearby results [21-24].

Similarly, the neutrophils CD11b expression levels were significantly high in patients with neonatal sepsis with sensitivity, specificity, PPV and NPV of 95%, 95%, 94.1% and 95.9%, respectively. These results favorably agree with those reported by Umlauf and his coworkers (2013), who reported a similar high sensitivity (96%) of CD11b for detection of neonatal sepsis [25]. That study has shown that about 95% of the total content of the resting neutrophils CD11b is in the form of intracellular storage vesicles, with only 5% of CD11b expressed on cell surface. Upon stimulation of the neutrophils, exocytosis of these secretory vesicles occurs with consequent enhanced CD11b expression [26, 27].

Serum and urinary NGAL have been shown to be an ideal biomarker of bacterial infection and systemic inflammation in both children and adults [28]. The result of lipocalin-2 for diagnosing neonatal sepsis was very rewarding. Its level was significantly high in the presence of infection. Moreover, the marker had high sensitivity, specificity (90% & 95% respectively). The only shortcoming of Lipocalin-2 is its relatively low negative predictive value (82.6%) for detection of neonatal sepsis. However, serial measures may improve the sensitivity and negative predictive value of this biomarker. Khosravi and his coworkers reported a similar high sensitivity (92%) and specificity (91%) of serum lipocalin-2 for detection of neonatal sepsis [29]. Other investigators previously found that lipocalin-2 was significantly higher in the infected group (mean 587.6 µg/l) than in the non-proven infected group (mean 217.7 µg/l, $P < 0.001$) and its level was peaked one day earlier than CRP [30].

In comparisons to CRP, the CD11b, CD64 and lipocalin-2 have remarkably higher sensitivities and specificities for

diagnosis of neonatal sepsis. This could be attributed to the non-specific nature of CRP. In the present study, CRP has a sensitivity of 73% and specificity of 85.4% for diagnosis of neonatal sepsis. These rates coincide with the results of many previously published studies [31-33]. It has been proposed that CRP is a late rather than early marker of neonatal sepsis and serial quantitative measurements at 24 and 48 hours after the onset of sepsis considerably improve the sensitivity [34].

The present study strongly supported the use of neutrophils CD64, CD11b and lipocalin-2 as biomarkers for neonatal sepsis; however, there were some limitations. The main limitation was the relatively small sample size of the participants. This was attributed to the financial constraints at Sohag University hospital. Another limitation was the use of blood culture as the gold standard test for diagnosis sepsis regardless its false positive and negative results. Another limitation was the assessment of total NGAL; secreted by the activated neutrophils. The measurement of monomeric NGAL which is secreted by injured kidney tubular cells, a frequent sequel of sepsis, could be more accurate [35]. Despite these limitations, this study paved the way for introduction of the studied markers for early diagnosis of neonatal sepsis. This strategy, if popularized, could hasten the early detection of neonatal sepsis and consequently help to decrease the neonatal mortality and morbidity in our locality as well as might help to save some resources which are being lost with the use of expensive unnecessary antibiotics. However, before its implementation, the results of the current study should be reappraised by another large scale study.

In conclusion, neutrophils CD64, CD11b and lipocalin-2 are highly sensitive and

specific markers of neonatal sepsis. These markers are superior to C-reactive protein for diagnosis of neonatal sepsis in terms of higher sensitivity and specificity, and to blood culture in terms of early reproduction of the results with minimal blood volume which is a real merit in the neonates.

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