CD$_{64}$ Cell Surface Expression on Neutrophils For Diagnosis of Neonatal Sepsis

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Neonatal sepsis (NS) continues to be one of the most significant causes of neonatal morbidity and mortality. Early identification of Neonatal sepsis is a major diagnostic problem because of the nonspecific clinical signs and limitations of the current diagnostic procedures. Neutrophil CD$_{64}$ expression has been proposed as a diagnostic test for evaluation of infection and sepsis. We compared the diagnostic utility of neutrophil CD$_{64}$ expression with IL-6, IL-8, TNF$\alpha$ and CRP assays. Peripheral blood samples were taken from 25 neonates classified into two groups; proven NS (n=15), clinical NS (n=10) and healthy newborns (n=10). CD$_{64}$ expression was analysed by flowcytometry, while serum level of interleukins (IL-6, IL-8), and TNF$\alpha$ was determined by ELISA. Expression of CD$_{64}$ was significantly enhanced in the groups with proven sepsis and clinical NS as compared to the controls ($P<0.05$). Similarly, TNF$\alpha$, IL-6, IL-8 and CRP levels were significantly elevated in the groups with sepsis and clinical NS as compared to the controls ($P<0.05$). Our data indicate that, in addition to serum levels of interleukins (IL-6, IL-8), and TNF$\alpha$, expression of CD$_{64}$ on neutrophils by flowcytometry could be useful as an indicator of NS due to its early appearance, sensitivity and specificity (96%). In conclusion, neutrophil expression of CD$_{64}$ is a useful diagnostic tool for early detection of neonatal sepsis. The assay is rapid, easy and reliable.

Neonatal sepsis (NS) is a clinical syndrome of systemic illness accompanied by bacteremia occurring in the first month of life (Gomella et al., 2004). NS remains as a major cause of morbidity and mortality in the newborn, mainly among preterm and low birth weight infants. Surviving infants can have significant neurologic sequelae as a consequence of central nervous system involvement, septic shock or hypoxemia (Betty & Inderpreet, 2005). Infection in the neonates presents diagnostic dilemma as the clinical presentation is non-specific and there’s no single reliable test for the early confirmation of definite sepsis (Santana et al., 2003). Most infants with septicemia are presented with non specific signs and symptoms including temperature instability, lethargy, apnea and poor feeding, late manifestations include convulsions and thrombosis (Bang & Reddy, 2005). Culture of body fluids particularly blood and cerebrospinal fluid is regarded as the gold standard for the diagnosis of infection, but final culture results are usually not available until at least 48-72 hrs after collection (Mehr & Doyle, 2001).

Cytokines are intracellular signaling polypeptides produced by activated cells during an episode of sepsis (Gabay & Kushner, 2002). The research for diagnostic tests for sepsis in newborn infants has turned to cytokines as well as to other chemical substances associated with the inflammatory response in some cases induced by cytokines, as possible indicators of infection (Chiesa et al., 2004). The lack of a single highly specific and sensitive laboratory indicator of acute inflammation has resulted in the clinical use of several concurrent laboratory tests in routine practice that indicate infection or significant acute inflammatory response (Bruce et al., 2006).

CD$_{64}$ is a member of the Fc gamma receptor I family of cell surface proteins that play an important role in both host defense
and autoimmune disorders (Pan et al., 1999). The Fcγ receptor (CD64) is a high affinity receptor normally expressed by monocytes and involved in phagocytosis and intracellular killing of bacteria. Neutrophils from healthy individuals express CD64 to a very low extent. During bacterial infections, however, the expression of this receptor increases markedly on neutrophils (Van der Meer et al., 2007). The CD64 behave as activation antigens in neutrophils, increasing their expression on the cell surface after leucocyte activation. Therefore, it is expected that during NS, bacterial products stimulate the release of IL-6 and TNF-α, inducing the activation of neutrophils and resulting in changes in the expression of leucocyte-differentiation antigens, with an increase in CD64 levels (Fjaertoft et al., 2005). Flowcytometry allowed simultaneous measurement of key markers using only minimal blood volume. Selection of markers with complementary properties could greatly increase the ability of neonatologists to diagnose infection (Ng & Lan, 2006).

This study was performed to develop a simple, reliable immunological indicator for diagnosis of neonatal sepsis.

**Patients and Methods**

**Subjects**

Neonates, who were admitted to the neonatal intensive care unit at Al Zahraa University hospital were included in the study. They were classified into the following two groups:

Group I consisted of 15 preterm newborn infants with obvious clinical signs of infection and positive bacterical blood culture (proven sepsis). Their gestational age ranged from (37-39 wks), their weight ranged between (2.5-3.5 kg), their sex are 10 males, 5 females. 7 of them were born by caesarian section, 8 were born by normal vaginal delivery.

Group II included 10 neonates with negative bacterical culture but have three or more clinical signs of infection such as fever, temperature instability, tachycardia, tachypnea, abdominal distention, respiratory distress, seizures, apnea, cyanosis, oliguria, gastrointestinal bleeding or petechiae. Their gestational age ranged from (37-39 wks), their weight ranged from (2.8 – 3.7 kg) 6 were males and 4 females, 6 of them were born by normal vaginal delivery and 4 C- section.

Group III included 10 manifested neonates with matched gestational age as control group. They were under investigation because of a suspicion of different diseases, but all had normal results and no illness was subsequently detected.

**Blood Samples:**

The study was approved by the ethical committee of Alzahraa University Hospital, blood samples were collected after informed consent from the parents.

Samples of peripheral blood were obtained from all cases during the first 24 hrs of clinical suspicion of NS. A single sample (5 ml) was obtained in sterile tube simultaneously with that taken for bacterial culture and complete blood count (CBC). Samples for group 3 (control group) were taken at the time of blood drawn for laboratory testing and within the first 48 hrs of their admission.

Blood samples were immediately transported to the laboratory, and they were processed upon arrival; serum was obtained from blood by clotting then centrifugation and stored at -20°C for subsequent quantification of cytokines (IL-6, IL-8 and TNFα), the rest of blood sample was taken in tube containing EDTA to detect the expression of leucocyte-differentiation antigen by flowcytometry.

Patient and control groups were subjected to the following:

- History taking to identify the presence of a risk factor for infection stressing on gestational age, birth weight, mode of delivery, premature rupture of membrane (PROM) and maternal fever.
- Complete clinical examination to detect the signs of neonatal infection.
- Laboratory investigations included:
  - Complete blood count.
    The total leucocytic count, HB and platelet counts were measured on an automated celtac counter.
  - Semi-quantitative C-reactive protein (CRP):
    Determined by latex agglutination technique. It was considered positive when the titer was ≥ 6 mg/l (Mathers & Pohlandi, 1987).
  - Determination of IL-6, IL-8, TNFα. They were done for all cases and controls by enzyme-linked
immunosorbent assay (ELISA) using reagent kits from bender medsystems GMBH Campus Vienna Biocenter 2 A-1030 Vienna, Austria, Europe.

- Expression of CD$_{64}$ by neutrophils was analyzed in whole blood using flow cytometry, staining was performed using 50µl was incubated for 10 min. at room temperature with Fluorescein isothiocyanate (FITC)- conjugated anti-CD$_{64}$ mouse monoclonal antibody (Cymbus biotechnology). The erythrocytes were lysed by 1.5 ml of lyse solution (NH$_4$ Cl buffered with KHCO$_3$ at pH 7.2). Neutrophils were electronically selected on the basis of their side – and forward – scatter characteristics and 10,000 cells were analyzed in each sample. Results were expressed as a percentage of positive cells.

**Statistical Analysis**

Data were expressed as the arithmetic mean ± SD. Differences amongst the groups were tested using Mann-Whitney-U-test, statistically significant differences were defined as $P<0.05$. The receiver operator characteristic (ROC) curve was obtained to estimate the best cut off value for studied parameters, its specificity, sensitivity, positive predictive value (PPV) and negative predictive value (NPV).

**Results**

A total of 35 neonates studied 15, 10 and 10 of whom belonged to the infected group I (proven sepsis), (clinical sepsis) group II, and (healthy newborns) group III respectively. There were no significant differences among the groups regarding gestational age and birth weight ($P > 0.05$) (Table 1).

Table (2) compares serum level of IL-6, IL-8, TNF$_{a}$, CRP concentration and neutrophil CD$_{64}$ expression between patient groups. In contrast serum levels of IL-6, IL-8, TNF$_{a}$, CRP and CD$_{64}$ their were no significant difference between proven sepsis and clinical sepsis.

| Table 1. Demographic characteristics of neonates with sepsis and controls. |
|-----------------------------|-----------------------------|-----------------------------|
|                             | Group I (Proven sepsis)     | Group II (Clinical sepsis)   | Group III (Healthy newborns) |
| No of newborns              | (15)                        | (10)                        | (10)                        |
| Gestational Age (WK)        | 38.66 ± 1.64                | 38.85 ± 1.53                | 38.90 ± 1.15                |
| Birth weight (kg)           | 3.22 ± 0.41                 | 3.61 ± 0.61                 | 3.41 ± 0.42                 |
| Male: Female                | 10 (66.6%): 5 (33.4%)       | 6 (60%): 4 (40%)            | 6 (60%): 4 (40%)            |

| Table 2. Comparison between bacteriologically proven and clinical sepsis patients as regard serum level of IL-6, IL-8, TNF$_{a}$, CRP and CD$_{64}$. |
|-----------------------------|-----------------------------|-----------------------------|
|                             | Group I (Proven sepsis)     | Group II (clinical sepsis)   | $P$ value |
|                             | (n = 15)                    | (n = 10)                    |            |
| IL-6 (pg/ml)                | 429.40 ± 116.95             | 470.00 ± 53.78              | NS         |
| IL-8 (pg/ml)                | 1.94 ± 1.25                 | 3.08 ± 1.33                 | NS         |
| TNF$_{a}$ (pg/ml)           | 45.00 ± 19.91               | 54.50 ± 14.57               | ND         |
| CRP (mg/l)                  | 24.00 ± 13.71               | 25.90 ± 12.91               | NS         |
| CD$_{64}$(%)                | 76.66 ± 22.78               | 80.98 ± 135                 | NS         |

$P>0.05$ is not significant. NS=not significant.
Table (3) compares serum level of IL-6, IL-8, TNFα, CRP concentration and neutrophil CD64 expression between patients group and control group. The serum level for all parameters were significantly higher in patients group compared to control group ($P<0.001$).

<table>
<thead>
<tr>
<th>Patients group (n = 25)</th>
<th>Control group (n = 10)</th>
<th>$P$ value</th>
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<tbody>
<tr>
<td>IL-6 (pg/ml) 445.64 ± 97.34</td>
<td>7.90 ± 3.03</td>
<td>0.001</td>
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<tr>
<td>IL-8 (pg/ml) 2.35 ± 1.37</td>
<td>0.27 ± 0.14</td>
<td>0.001</td>
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<tr>
<td>TNFα (pg/ml) 48.80 ± 18.26</td>
<td>11.26 ± 3.41</td>
<td>0.001</td>
</tr>
<tr>
<td>CRP (mg/l) 24.76 ± 13.15</td>
<td>0.5 ± 2.1</td>
<td>0.001</td>
</tr>
<tr>
<td>CD64 (%) 78.39 ± 19.32</td>
<td>3.38 ± 2.23</td>
<td>0.001</td>
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$P<0.05$ is significant.

Table (4) shows that IL-6 serum levels have a high diagnostic specificity for NS (90% ROC curve analysis). The diagnostic sensitivity, and the positive and negative predictive values for the cytokines were 80%, 78% and 75% respectively at cut off 11. IL-8 diagnostic specificity for NS (85% ROC curve analysis). Diagnostic sensitivity, positive and negative predictive value 90%, 78.6, 80 respectively at cut off 0.55. The sensitivity and specificity of raised serum level of TNFα, for diagnosis of neonatal sepsis were 78% and 80%, and positive and negative predictive values were 90.13% and 88.89% respectively at cut off 10.5. CRP diagnostic specificity for NS (90% ROC curve analysis). Diagnostic sensitivity, positive and negative predictive value 95%, 96.15, 90.5 respectively at cut off 6. As regard CD64 diagnostic specificity for NS (96% ROC curve analysis). Diagnostic sensitivity, positive and negative predictive value 95%, 96%, 90% respectively at cut off 5.

Figure (1) showed the percentage of positive cells by flowcytometry analysis of CD64 expression on neutrophils from patient with neonatal sepsis.

<table>
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<tr>
<th>Table 4. Comparison of sensitivity, specificity PPV and NPV of markers (IL-6, IL-8, TNFα, CRP and CD64).</th>
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<tr>
<td>IL-6 (%)</td>
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<tr>
<td>Sensitivity</td>
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Discussion

Because of the nonspecific signs, the clinical diagnosis of sepsis in neonates is difficult to determine. Rapid diagnosis is problematic because the first signs of this disease may be minimal, and are similar to those of various non-infectious processes; furthermore, bacterial cultures are time-consuming, and other laboratory tests are either not available for routine use or lack sensitivity or specificity. In this situation, neonates with risk factors for infection or clinical suspicion of infection are empirically treated with antibiotics. To avoid the unnecessary treatment of an infected patients, an early, sensitive and specific laboratory test would be helpful to guide clinical clinicians in neonatal units in deciding whether or not to start administering antibiotics (Livadili et al., 2006).

As it is not easy clinically to diagnose sepsis in newborn, several groups of researchers have attempted to characterize a laboratory indicator that is useful for the early diagnosis of NS. Some immunological markers such as serum levels of IgG, IgM, C3 and C4 have been evaluated, but they did not seem to have significant value for the early diagnosis of NS (Kalayci et al., 1997). In addition, it has found that the serum levels of soluble receptors of TNF only show small variations in NS, and useful as indicators of this condition (Layseca- Espinosa et al., 2002).

Several leukocyte indices and acute-phase protein levels have been evaluated for the diagnosis of sepsis and measurement of multiple plasma cytokines (Hodge et al., 2004a), and leukocyte activation markers (Hodge et al., 2004b) have showed promising results. Non specific, diagnostic-screening tests helping the clinician to identify newborns suffering from early onset sepsis (EOS), acute phase reactants such as C-reactive protein (CRP) and cytokines, for example, interleukin, IL-6, IL-8, and tumor necrosis factor alpha (TNFα), have been shown to correlate with the diagnosis of EOS (Gerdes, 2004).

However, CRP appears to be less sensitive at onset of sepsis and cytokines owing to their short half-life are less sensitive after 12 - 24 h
of onset of sepsis, thus increasing the risk of an early laboratory marker with high diagnostic sensitivity and specificity for NS would be a valuable tool for therapeutic decision-making, thus avoiding the unnecessary use of antibiotics in those patients without infection but in whom sepsis is suspected on a clinical basis (Volante et al., 2004).

In the present study the kinetics of neutrophil membrane CD$_{64}$ expression were examined in 25 neonate suffering from sepsis using flowcytometry and compared its results with CRP, cytokine (TNF$\alpha$) IL-6, and IL-8. In this study, we attempted to identify a novel immunological indicators that, through a single and rapid determination, were useful for the diagnosis of NS.

Proinflammatory cytokine biomarkers commonly associated with sepsis, TNF$\alpha$ and IL-6 which are implicated in the early host response to infection; increased concentrations have been demonstrated by several studies on septic neonates (Carrigan et al., 2004). In this study, it has been found that serum concentration of IL-6, IL-8, TNF$\alpha$ were significantly highly elevated in septic newborn at the time of admission to the neonatal intensive care unit comparing the mean level with that of the control group ($P<0.001$). This finding agree with Preocianoy, (2004) who found that there were increase in these immune parameters which are sensitive indicators of systemic infection.

The disparity between the high diagnostic sensitivity reported in this study for IL-6 and IL-8 and the values of Layseca-Espinosa et al., (2002), could be the result of differences at the time at which blood samples were taken, for IL-6, IL-8 have a short half-life in plasma. In this regard, Panera et al., (1999) made two separate determinations of IL-6 and plasma levels in each patient with NS, and found additional measurements are helpful for detecting infection in the neonate. It is important to note that in this study only one blood sample from each patient was obtained.

Sensitivity and negative predictive values for IL-6 being 80% and75% but Smulian et al. (1999) on his study found that sensitivity and negative predictive values being 87% and 93% respectively Khairallah et al., (2005) reported that IL-6 was a potent proinflammatory cytokine and responsible for eliciting a strong inflammatory reaction, IL-6 level was elevated many folds in infected neonates compared to non infected one.

In the present study CRP ($\geq$6mg/L) had a sensitivity 95% and negative PV of 90.5% specificity 90% positive PV of 97.42%. Bentiz et al., (2001) reported a sensitivity of 35% for quantitative CRP assay at cut off level 6 mg/L while Nuntarumit et al., (2002) stated that at cut off level 6mg/L sensitivity of CRP quantitative assay was 100% specificity was 94% to PV was 91.6% and negative PV was 100%. He mentioned that predictive value should be enhanced by serial rather than single measurement. Result of this study disagree with Ng et al. (2002) they found that sensitivity and negative PV for IL-6 and CRP were 100%, specificity and positive PV were exceeding 88% and 80%.

There was a statistically significant difference between patient groups and control group for serum level of IL-8 which being higher in patient group when compared with control group, $P$ value was highly significant ($P<0.001$). This was in agreement with Santana et al., (2003) and Orlikowsky et al., (2006), they found that IL-8 was significantly increased in patient groups compared to the control group. In this study sensitivity, specificity positive, negative PV for IL-8 were 90%, 85%, 78.6%, 88.13%, respectively, result obtained by Martin et al., (2001) sensitivity was 70%, specificity was 92% the positive PV was 93% the negative PV was 65% this disagree with the result of this study due to different cut off value between us. Ng & Lan (2006) found in their study that serial
measurement and use of combinations of these tests (IL-6, IL-8, CRP) have been reported to improve sensitivity and negative predictive value of these tests.

In this study correlation between IL-6 and the age, birth weight, as well as other inflammatory parameters namely CRP found no statistical significant correlation between them \((P>0.05)\) this was in agreement with Ozdemir \textit{et al.}, (2004) who could not find statistical significant correlation between any of the cytokine levels, and CRP in cases with neonatal infection.

Franz \textit{et al.} (2001) found that the combination of IL-8 with CRP is a reliable test for the diagnosis of early onset bacterial infection and may be helpful in enabling antibiotic therapy to be reduced in newborn infants. IL-6 is the main stimulus involved in the induction of the acute-phase reaction and enhancement of C-reactive protein (CRP) synthesis, (it preceedes the increase in CRP), Ng & Lan (2006) found in their study that serial measurements and use of combinations of these tests (IL-6, IL-8, CRP) have been reported to improve sensitivity and negative predictive value of these tests.

In this study we attempted to identify a simple immunological indicator of NS that was useful with a single and rapid determination. The analysis of expression of CD\textsubscript{64} provides a reliable and prompt result for the clinician. This analysis can be combined with additional, time consuming determination, such as ELISA measurements of TNF\(\alpha\), IL-6, IL-8 to improve the probability of NS detection. The results of this study showed that the enhanced expression of CD\textsubscript{64} by neutrophils has a high specificity. The high specificity of raised levels of CD\textsubscript{64} for NS is achieved through a single determination of CD\textsubscript{64} in NS. In our result CD\textsubscript{64} sensitivity and specificity were 95\%, 96\% respectively, this finding agree with Ng \textit{et al.} (2002), they found that CD\textsubscript{64} has highest sensitivity (97\%) and negative PV (99\%), and also Nautila \textit{et al.} (2007) found in their study that sensitivity was 94\% and specificity was 98\% so CD\textsubscript{64} expression analysis is a useful marker of infection. Layseca-Espinosa \textit{et al.}, (2002) in their study found that CD\textsubscript{64} diagnostic specificity was 96.8\% which agree with our result.

In this study, no significant correlation was found between any cytokine, CRP and CD\textsubscript{64} in cases with neonatal infection. These findings are in disagreement with Layseca-Espinosa \textit{et al.} (2002), who demonstrated a significant correlation between IL-6 plasma level and CD\textsubscript{64} expression but not between TNF\(\alpha\) level and CD\textsubscript{64}. This may be due to differences in the kinetics of cytokine synthesis, neutrophil activation and neutrophil half-life.

In conclusion, it is very important to develop an early reliable indicator of neonatal sepsis to provide immediate and avoid unnecessary therapy. IL-6 and IL-8 can not be used alone for early diagnosis of NS. CD\textsubscript{64} expression may provide additional assistance in the early management of neonates with suspicion of NS. In such cases, a high expression of CD\textsubscript{64} would strongly suggest the presence of infection and indicate that antibiotic administration is necessary.

References

6. Davis BH, Olsen SH, Nancy C, Bigelow (2006). Neutrophil CD_{64} is an improved indicator of infection or sepsis in emergency department patients. Archives of pathology and laboratory medicine; 130(5): 654-61.


