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ORIGINAL ARTICLE

# Flow Cytometric Evaluation of CD11b Expression on Peripheral Blood Neutrophils for Early and Rapid Diagnosis of Neonatal Sepsis

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***Neonatal sepsis, CD11b, flow cytometry, CRP, Blood culture***

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## ABSTRACT

***Background****: Neonatal sepsis is life-threatening disease; it represents a big problem duo to its difficult diagnosis. It is associated with high mortality rate if not treated promptly. It is therefore very critical to do early diagnosis and initiate adequate therapy.* ***Objective*** *was to make a flow cytometric evaluation of CD11b expression on peripheral blood neutrophils for early and rapid diagnosis of neonatal sepsis.* ***Methodology:*** *our study was carried out on 70 neonates and subdivided into 2 groups, study group consisted of 50 neonates and control group consisted of 20 apparent healthy neonates, three ml of blood were collected for evaluation of CD11b expression by flow cytometry, CBC, CRP determination, and for blood culture.* ***Results:*** *there was a significant increase in percentage expression of CD11b on peripheral blood neutrophils in group I (sepsis group) more than group II (control group) , CD11b expression level in % on peripheral blood neutrophils record the highest sensitivity and specificity for diagnosis of neonatal sepsis followed by CRP. Blood culture recorded the least sensitivity and specificity.* ***Conclusion:*** *flow cytometric detection of CD11b on peripheral blood neutrophils is a useful technique for early and rapid diagnosis of neonatal sepsis.*

## INTRODUCTION

Neonatal sepsis is a highly inflammatory disease that results in a septic shock and failure of many organs. Also can be defined as a clinical syndrome characterized by systemic signs of circulatory compromise such as poor peripheral perfusion, pallor, hypotonia and poor responsiveness caused by bacteria that invade the bloodstream during the first month of life. It is a life-threatening disease that represents an important cause of morbidity and mortality in the neonate[1].

Neonatal sepsis can be classified into early-onset and late-onset sepsis. Early onset neonatal sepsis occurs before the first 4 to 7 days of life and is usually caused by microorganisms transmitted from mother to her infant. The infant acquires the organism during the intrapartum period from the maternal genital tract. Late onset neonatal sepsis is acquired postnataly from an environmental source[2].

Causative organisms of early-onset neonatal sepsis are *Group B Streptococcus, Escherichia coli, Coagulase-negative Staphylococcus, Haemophilus influenzae, listeria monocytogenes* and for late-onset neonatal sepsis are *Staphylococcus aureus, Enterobacter, Pseudomonas, Klebsiella, E coli* [3].

Neonatal sepsis diagnosis is a very difficult problem in clinical medicine, because of its nonspecific clinical symptoms and signs. Causative microorganism isolation by blood culture was the gold standard for neonatal sepsis diagnosis [4].

However, blood cultures have many disadvantages such as delay in the diagnosis and low sensitivity .These disadvantages were duo to intermittent appearance of causative microorganisms in the blood stream, the very small blood volumes could be obtained from infant for culture and the habit of giving mothers especially those of high risk deliveries intrapartum antibiotics. Also, a negative blood culture occurs in case of pneumonia and meningitis [5]

Neonatal sepsis diagnosis could be done by White blood cell count, but it also has many disadvantages such as it is nonspecific method and has low positive predictive value. Many factors can affect it such as infections, age of infant in hours, blood sampling method, delivery method, and hypertension of the mother and the sex of the infant [6].

Complete blood counts and hematological score which involves seven findings significantly associated with sepsis are Abnormal total leukocyte count, abnormal total PMN count, elevated immature PMN count, elevated immature to total (I:T) PMN ratio, immature to mature(I:M) PMN ratio greater than or

equal to 0.3, platelet count less than or equal 150,000/mm3 , and pronouced degenerative or toxic changes in PMNs. The likelihood of sepsis is with score greater than or equal to 3 also used for diagnosis of neonatal sepsis [7].

C-reactive protein (CRP), which is a major acute phase plasma protein synthesized by hepatocytes, is of limited use in early diagnosis of neonatal sepsis .It increases within 24 hours of infection but remains normal at the onset and after 8 hours of infection. This lag period between the triggering factors and the onset of a positive CRP result makes a single CRP measurement of very limited value in early diagnosis of neonatal sepsis. However, Serial CRP measurements may be useful for follow up the course of sepsis and the treatment response [8].

CD11b is a surface antigen of neutrophil that is expressed normally at a very low concentration on the surface of non-activated neutrophils. It increases on the neutrophil surface within 5 minutes in response to exposure to bacteria or endotoxin which could make it a sensitive and specific method for early and rapid diagnosis of neonatal sepsis. Early diagnosis of neonatal sepsis is very important to inmate treatment of neonatal sepsis rapidly and thus reduces the high mortality and morbidity[9].

CD11b expression on peripheral blood neutrophils can be detected using a fluorescent activated cell sorting flow cytometer with monoclonal antibodies recognizig CD11b[10]. It is a fast technique that provides great detailed data regarding the cells and is less expensive than other used techniques such as direct immunofluorescence[11]. The aim of our study was to make a flow cytometric evaluation of CD11b expression on peripheral blood neutrophils for early and rapid diagnosis of neonatal sepsis.

## METHODOLOGY

The present study was carried out on neonates admitted to Neonatal Intensive Care Unit of Tanta University Hospital.

The subjects were subdivided into 2 groups, Group1 (study group): consists of 50 neonates suspected to have neonatal sepsis and Group2 (control group): consist of 20 apparent healthy neonates.

Inclusion Criteria were neonates who showed evidence of acute clinical deterioration such as (fever, grunting, apnea, cyanosis, tachycardia, hypotonia) or had a risk factor for infection such as[12]: Premature rupture of membrane, Pre-partum maternal fever, maternal antibiotics, Pre-maturity, Presence of foreign body (central venous catheter, endotracheal tube, chest tube, and umbilical catheter). Exclusion Criteria were neonates who were receiving antibiotic therapy [13].

The neonates included in this study were subjected to the following: Complete history taking (Prenatal, natal, and post natal history to detect risk factors), Complete clinical examination, clinical signs of sepsis will be searched for as: Respiratory dysfunction (apnea, grunting, intercostals retraction, cyanosis), Neurological dysfunction(irritability, hypotonia, lethargy, convulsion), GIT dysfunction(abdominal distension, hepatomegaly, feeding problems, jaundice), Cardiovascular dysfunction(tachycardia, bradycardia, poor perfusion, shock) and temperature instability (hypothermia, hyperthermia)

**- Laboratory investigation:**

***Immunological and hematological investigation:***

Two ml of blood were collected under complete aseptic precaution where one ml was added to EDTA for evaluation of CD11b expression by flow cytometry and for complete blood counts (CBC).The other one ml of blood was centrifuged in order to separate serum for CRP determination, samples were transported as soon as possible to be processed within 1 to 3 hours after sampling. CRP was done using latex agglutination test.

CBC: hematologic scoring was done according to hematologic scoring system [14]. Hematological sepsis scoring system ≥ 3 (total score 7, score ≥ 3 strongly suggestive to sepsis).These data were taken from the sheets of the patients and depended on parameters mentioned in table (1).

**Table 1: Hematological scoring system.** [14]

| *Criteria* | *Abnormality* | *Score* |
|---|---|---|
| **Total WBC count** | ≤5000/mm$^3$ | 1 |
| | ≥25000 at birth<br>≥30000-12-24 h<br>≥21000-day 2 onwards | 1 |
| **Total PMN count** | 1800-5400 | 0 |
| | No mature PMN seen | 2 |
| | Increased/decreased | 1 |
| **Immature PMN count** | 600 | 0 |
| | Increased | 1 |
| **I:T PMN ratio** | 0.120 | 0 |
| | Increased | 1 |
| **I:M PMN ratio** | ≤0.3 | 0 |
| | ≥0.3 | 1 |
| **Degenerative changes in PMN** | Toxic granules/cytoplasmic vacuoles | 1 |
| **Platelet count** | ≤150000/mm$^3$ | 1 |

WBC - White blood cells; PMN - Polymorph nuclear neutrophils; I:T PMN ratio - Immature: Total ratio; I:M PMN ratio - Immature: Mature ratio.

**- CD11b detection method[15]:** Steps were performed according to manufacturer instruction.

***Principle of the test:*** Cell surface expression of CD11b by polymorph nuclear leucocytes were determined by flow cytometric analysis using Monoclonal Mouse IgG2B Clone #238446 with 488nm wave length laser excitation. Cells expressing CD11b have the ability to

be fluorescently stained with intensity directly proportional to the density of expression of CD11b.

***Sample staining:***

1. For each sample, 2 tubes were labeled, one for monoclonal antibodies used, the other tube for negative isotype control.
2. 50 $\mu L$ of samples (whole blood on EDTA) were delivered in each tube.
3. 10 $\mu L$ of monoclonal antibodies were added to respective tubes.
4. The tubes were vortexed and incubated in the dark at $4^{o}C$ for 25 minutes.
5. 1.5 ml of lysing solution was added to each tube to lyse RBCs.
6. The tubes were vortexed and incubated for 20 minutes in the dark at $4^{o}C$.
7. The tubes were centrifuged at 3000 rpm for 5 minutes and supernatant was discarded.
8. 3 ml of PBS as washing solution was added to each tube and mixed thoroughly.
9. The tubes were centrifuged at 3000 rpm for 5 minutes and the supernatant was discarded, this step was repeated twice.
10. Cell pellets were suspended in 300 $\mu L$ PBS to be ready for acquiring data by the flow cytometer.

***Flow cytometric analysis:***

1. The full alignment procedure was performed using standard calibride beads for adjusting forward scatter, side scatter and photomultiplier tube (PMT).
2. Control samples were introduced in the machine and forced in the sheath by the sample pressure (run button) where the laser scatter was received on both forward scatter detectors and the scale to show the cell population in a basic histogram and to adjust the regions.
3. 10000 events (cells) at least were passed in front of the laser for each case.
4. The sample tubes were then introduced and processed in the same way as the control, where the cells tagged with monoclonal Phycoerythrin (PE)(is a red protein-pigment complex from the light-harvesting phycobiliprotein family, present in red algae and cyanobacteria, responsible for photosynthsis) antibody were analyzed.

- **Bacteriologic investigation:**

One ml of blood was collected under complete aseptic precautions to be immediately inoculated into blood culture bottles, blood culture bottles were transported as soon as possible to be incubated aerobically at 37c, the bottles were observed daily up to seven day for signs of bacterial growth such as turbidity, gas production, and haemolysis, bottles suspected for bacterial growth were subcultured on different solid culture media (blood agar, MacConkey agar, chocolate agar, and nutrient agar), colonies were examined for colony morphology and Gram stain was done. In case of absence of growth signs, blind subculture was done after 24 hours over the different solid media and another subculture was done before reporting the sample as negative.

## RESULTS

Our study was carried out on neonates admitted to Neonatal Intensive Care Unit of Tanta University Hospital.

The subjects were subdivided into 2 groups, Group1 (study group): consists of 50 neonates suspected to have neonatal sepsis and Group2 (control group): consist of 20 apparent healthy neonates, in group I 52% were males and 48% were females. On the other hand, group II were 55% males and 45% females, as regard the mode of delivery Group I showed that 50% were delivered through normal vaginal delivery (NVD) and 50% through caesarian section (CS). On the other hand, group II showed that 50% were delivered through normal vaginal delivery (NVD) and 50% through caesarian section (CS), the mean gestational age ± SD of the group I was 36.64 weeks ± 1.91 while the mean gestational age ± SD of the group II was 38.20 weeks ± 0.77. The mean weight ± SD of the group I was 2.68 ± 0.43 while the mean weight ± SD of the group II was 2.96 ± 0.28, there was no significant relation between sex or mode of delivery and neonatal sepsis but there was significant decrease in age in sepsis group more than control group and there was significant decrease in weight in sepsis group more than control group as shown in table 2.

As regard distribution of different risk factors for sepsis among group I (sepsis group) and group II (control group) P value was (0.001), (0.024) : means that there is significant increase in maternal history of (PROM), pre-partum fever, chorioamnionitis and incidence of prematurity in group I (sepsis group) more than group II (control group) as shown in table 3.

As regard results of flowcytometric measurement of CD11b on peripheral blood neutrophils there was significant increase in percentage expression of CD11b on peripheral blood neutrophils in group I (sepsis group) more than group II (control group) P value was (0.001) as shown in table 4.

As regard comparison between data of Receiver operating characteristic (ROC) curve of CD11b expression level in % on peripheral blood neutrophils, C-reactive protein (CRP) and blood culture used for diagnosis of neonatal sepsis. CD11b expression level in % on peripheral blood neutrophils record the highest sensitivity and specificity for diagnosis of neonatal sepsis followed by CRP. Blood culture record the least sensitivity and specificity as shown in table 5.

**Table 2: Gestational age(weeks) and weight among group I neonates (sepsis group) and group II neonates (control group).**

| | | Sepsis N=50 | Control N=20 | t. test | p. value |
|---|---|---|---|---|---|
| **G. age (weeks)** | Range | 30–39 | 37–39 | 12.395 | P1: 0.001* |
| | Mean ± S. D | 36.64±1.91 | 38.20±0.77 | | |
| **Weight** | Range | 1.25–3.3 | 2.6–3.6 | 6.996 | P2: 0.010* |
| | Mean ± S. D | 2.68±0.43 | 2.96±0.28 | | |

**Table 3: Distribution of different risk factors for sepsis among group I (sepsis group) and group II (control group).**

| | | | Sepsis N= 50 | Control N= 20 | $X^2$ | P-value |
|---|---|---|---|---|---|---|
| **PROM** | **Positive** | **N** | 36 | 2 | **22.128** | **0.001*** |
| | | **%** | 72.0% | 10.0% | | |
| | **Negative** | **N** | 14 | 18 | | |
| | | **%** | 28.0% | 90.0% | | |
| **Pre-partum maternal fever** | **Positive** | **N** | 31 | 1 | **18.703** | **0.001*** |
| | | **%** | 62.0% | 5.0% | | |
| | **Negative** | **N** | 19 | 19 | | |
| | | **%** | 38.0% | 95.0% | | |
| **Pre-maturity (< 37 weeks)** | **Positive** | **N** | 24 | 0 | **22.128** | **0.001*** |
| | | **%** | 48.0% | .0% | | |
| | **Negative** | **N** | 26 | 20 | | |
| | | **%** | 52.0% | 100.0% | | |
| **Chorioamnionitis** | **Positive** | **N** | 15 | 1 | **5.064** | **0.024*** |
| | | **%** | 30.0% | 5.0% | | |
| | **Negative** | **N** | 35 | 19 | | |
| | | **%** | 70.0% | 95.0% | | |

**Table 4: CD11b expression level in % on peripheral blood neutrophils among group I (sepsis group) and group II (control group).**

| | | Sepsis N= 50 | Control N= 20 | t. test | p. value |
|---|---|---|---|---|---|
| **CD 11 b %** | Range | 34–99.7 | 17–35 | 242.487 | 0.001* |
| | Mean ± S. D | 88.30±17.02 | 27.20±6.33 | | |

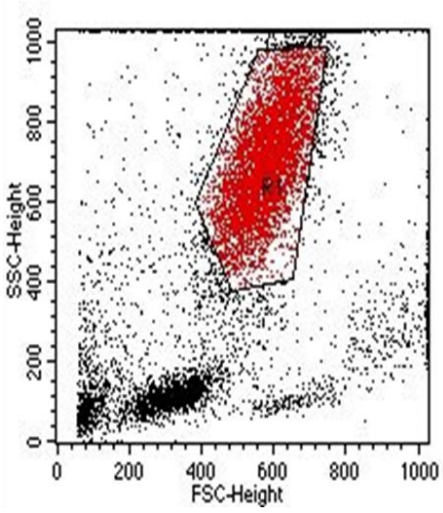


**Fig. 1: Dot plot expressing gating on granulocyte using Forward scatter (FS) versus side scatter (SS) strategy**

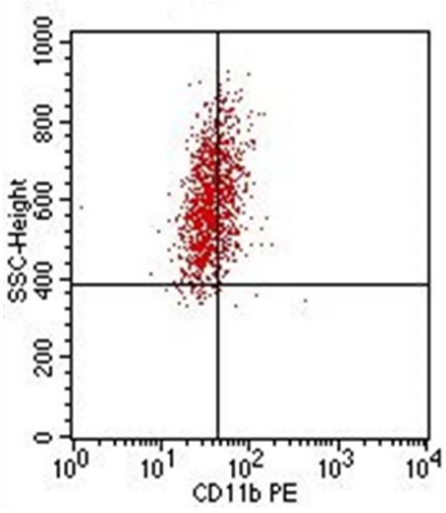


**Fig. 2: Dot plot showing weak expression of CD11b on granulocyte in the right upper quadrant**

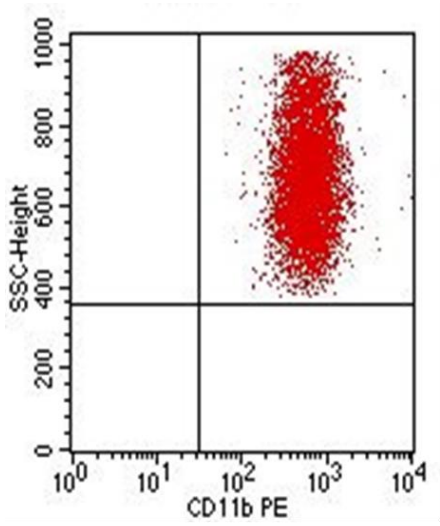


**Fig. 3: Dot plot showing strong expression of CD11b on granulocyte in the right upper quadrant**

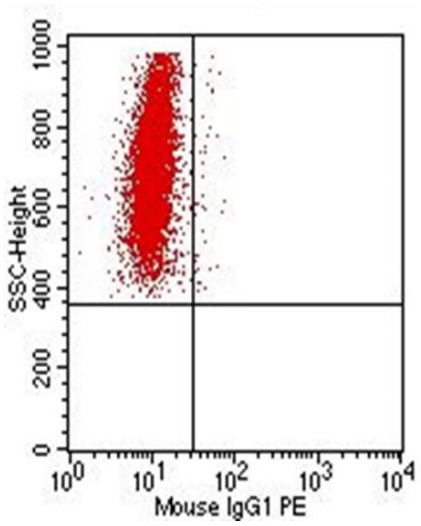


**Fig. 4: Dot plot showing negative isotype control of Phycoerythrin (PE)**

**Table 5: Comparison between data of Receiver operating characteristic (ROC) curve of CD11b expression level in % on peripheral blood neutrophils , C-reactive protein (CRP) and blood culture used for diagnosis of neonatal sepsis.**

| | AUC | Sensitivity | Specificity | PPV | NPV | Accuracy |
|---|---|---|---|---|---|---|
| **CD 11 b %** | 0.84 | 96 | 100 | 100 | 90 | 97 |
| **CRP** | 0.71 | 74 | - | - | 60 | 81 |
| **Blood culture** | 0.21 | 20 | Can't be determined | Can't be determined | 50 | 42 |

## DISCUSSION

Diagnosis of neonatal sepsis is important problem in clinical medicine, as there is no single reliable test for its early confirmation or exclusion. The gold standard for confirming the diagnosis of neonatal sepsis was isolation of the causal microorganism by blood culture[16].

However, blood culture techniques have many disadvantages such as delay the diagnosis and have low sensitivity[17]. An early biomarker of bacterial infection with high diagnostic sensitivity and specificity would be a valuable tool for therapeutic decision making, saving the life of many neonates and avoiding unnecessary use of antibiotics in those patients without infection but in whom sepsis is suspected on a clinical basis [18].

Our study was carried out on 70 neonates admitted to Neonatal Intensive Care Unit of Tanta University Hospital, 50 were suspected to have sepsis and 20 were used as control. It was performed in the Microbiology & Immunology Department and Clinical Pathology Department, Faculty of Medicine, Tanta University. The 20 neonates who were used as control were healthy full term neonates with physiologic hyperbilirubinemia, without symptoms and signs of sepsis.

It was found that the mean percentage expression of CD11b on peripheral blood neutrophils were significantly higher in sepsis group than in control group (88.30±17.02 versus 27.20±6.33).

Athis et al.[19], Genel et al.[20], Nupponen and Andersson,[21] and Adib et al.[22] found that the expression of CD11b on peripheral blood neutrophils were significantly increased in sepsis group more than control group. In contrast, Espinosa et al.[23] did not demonstrate a high elevation of CD11b in neonates with sepsis, which could be explained by differences in the genetic background of the patients studied which might affect both the basal expression of CD11b and the capability of neutrophils to respond to different bacterial stimuli.

On analysis of receiver operating characteristic (ROC) curve of CD11b expression level in % on peripheral blood neutrophils as a diagnostic test, it shows (At cutoff point 40): sensitivity of 96%, specificity of 100%.

In accordance, Volker et al. [15] found that both sensitivity and specificity of CD11b were 100%. Also, Nupponen et al. [20] found that the sensitivity of CD11b was 96% while specificity was 100%.

On the other hand, Cui and Cheng[24] showed that sensitivity and specificity of CD11b was 86.3% and

100% respectively, also Adib et al. [19] found that sensitivity and specificity were 75%, 100% respectively. This may explained by difference in accuracy of performance of used devices and different methods of categorization of studied cases.

The expression of CD11b does not differ with age, as its expression only occurs upon cell activation, thus this specific marker can be used for identification of the life threatening infection in preterm infants. Turnaround time of CD11b takes about less than 60 min; a blood volume of less as 100 µL is sufficient[19]. So, estimating CD11b expression level on peripheral blood neutrophils could be superior for blood culture in early diagnosis of neonatal sepsis and for starting empirical antibiotic therapy. This is due to rapidity of obtaining results, besides higher capability in ruling out infection especially in culture negative cases with avoidance of unnecessary use of antibiotics [25].

## CONCLUSION

Flow cytometric detection of CD11b on peripheral blood neutrophils is a useful technique for early and rapid diagnosis of neonatal sepsis.

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