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Evaluation of Neutrophil CD64 as a Marker in the Diagnosis of Neonatal Sepsis

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Abstract:

Background: *Neonatal sepsis is a global health problem due to its significant contribution to morbidity and mortality, particularly in developing countries. Diagnosis of neonatal sepsis remains a major challenge, as early signs of sepsis are often non-specific and the laboratory criteria are also not fully reliable. The blood culture is the most reliable method for detection of bacterial infections. But the sensitivity of blood culture is low, longer time required for final report and false negative result may be found.* ***Objective:*** *The objective of the*

study was to evaluate neutrophil CD64 as early diagnostic marker of neonatal sepsis. ***Methodology****: This cross sectional analytical study was carried out in the Department of Clinical Pathology, Neonatology and Microbiology & Immunology, BSMMU, Dhaka from March'2013 to February'2014. Non probability convenience sampling was used to collect 60 neonates in the study. There were 40 cases clinically diagnosed as neonatal sepsis and 20 control neonates with no symptoms or signs of infection were included in this study. Neutrophil CD64 expression was measured by Flow cytometry and blood cultures were done by Bactec microbial detection system (Becton Dickinson - 9240).* ***Result****: Mean±SD age of the study subjects was 5.9±6.49 days. Blood culture was found positive only in 9 (22.5%) cases. The result showed significantly elevated levels of CD64 in septic neonates (36.03 ±25.70) when compared to control group (4.85±2.95) also their percentage of expression was higher in culture positive sepsis (77.07±15.07) than culture negative sepsis (26.56±13.46). The study showed significant difference (P <0.001) between culture proven sepsis and culture unproven sepsis regarding percentage of expression of CD64 on neutrophil. Neutrophil CD64 showed high sensitivity, specificity, PPV and high NPV (100%, 54.9%, 28.13% and 100% respectively).So flow cytometric assessment of neutrophil CD64 may be considered as a rapid and reliable marker for the diagnosis of bacterial neonatal sepsis.*

Key words: Neutrophil CD64, Neonatal sepsis, Flow cytometry.

Introduction

Neonatal sepsis is a global health problem due to its significant contribution to morbidity and mortality[1]. In Bangladesh neonatal mortality rate is 32 per 1000 live birth[2]. Neonatal sepsis contributed 20% of neonatal death in Bangladesh[3]. There are two types of neonatal sepsis, early onset sepsis and late onset sepsis. Early onset of sepsis presents from birth to 72 hrs

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of life. Late onset sepsis is usually presents after 72 hours of age[4]. Diagnosis of neonatal sepsis is one of the most difficult tasks in clinical practice, as the disease progress more rapidly than adult and the mortality rate is higher in neonates[5]. Several different laboratory determinations are helpful in diagnosis of neonatal sepsis. Among them blood cultures are used as the gold standard for diagnosis of sepsis. It helps to make therapeutic decision, especially in choosing the appropriate antibiotics[6]. But culture results may be delayed for 24 hours for preliminary report to 7 days for final report after collection. The possibility of sepsis in the presence of negative blood culture is noted in neonates who are exposed to antibiotics in utero[1]. As a results of unnecessary exposure to antibiotics in neonates with clinical suspicion of sepsis, creates bacterial resistance[7]. The negative microbiological cultures do not always exclude the presence of bacterial sepsis[8]. So the other tests in diagnosis of neonatal sepsis are warranted. The white cell count, absolute neutrophil count (ANC), absolute band count, immature to total neutrophil ratio (I:T ratio), platelet count, micro-ESR and C-reactive protein (CRP) are still used as the screening test of neonatal sepsis. These tests are less sensitive and specific[9]. Thrombocytopenia with counts of <100 x109/l, increased mean platelet volume and platelet distribution width is noted in neonatal sepsis[10]. C - reactive protein (CRP) showed elevated in acute inflammatory diseases. Recently numerous cell surface antigens have been studied as promising biomarkers of infection, including CD11b, CD69 and CD64[11]. In flow cytometric technology neutrophil CD64 is found to be a promising marker for diagnosis of early and late infections in newborns. CD64 is a 72-kDa glycoprotein, known as FC gamma receptor 1 (FC γ RI). It binds immunoglobulin (IgG) with high affinity. The FC receptors, ligands immunoglobulin at constant regions, play a coordinating role in immunity and mediate functions such as endocytosis,

phagocytosis, antibody dependent cell mediated cytotoxicity (ADCC), and cytokine production[12]. CD64 is expressed on antigen presenting cells (monocytes, macrophages and dendritic cells), a lesser extend to eosinophils and only to a very low extent on resting neutrophils[13]. CD64 is highly correlated with the presence of infection and its expression can discriminate between acute inflammatory disease or systemic infections[14]. During neutrophil activation, inflammatory cytokines influence the upregulation of neutrophil CD64. Its expression is regulated in a graded fashion that is similarly parallel to the degree of inflammatory response or tissue injury[15]. Up-regulation of neutrophil CD64 occurs within hours[16]. Thus, the rapid up-regulation of CD64 expression on the neutrophil may provide a true indication of the current status of a patient's systemic acute inflammatory response to an infection. So the quantitation of the neutrophil CD64 may be a valuable sensitive and specific indicator of sepsis than the other available diagnostics tests. There are many advantages of using neutrophil CD64 expression as a diagnostic marker of neonatal sepsis. Up regulation of CD64 expression in activated neutrophil occurs within a relatively short time scale [4-6 hours][17]. The quantitation of neutrophil CD64 is rapid (below 1 hour) and only 100μL blood volume is used, which is a great advantage in neonates[1]. Detection of neutrophil CD64 may help in the early diagnosis of neonatal sepsis and may prevent unnecessary delay in diagnosis, enable prompt start of treatment and will help in reducing mortality and sepsis related complication. Another advantage is that neutrophil CD64 expression is not influenced by antibiotic therapy. Absence of any research in this field in our country has tempted me to undertake this study. Therefore, the purpose of the study is to evaluate the diagnostic sensitivity of neutrophil CD64 in the early diagnosis of neonatal sepsis.

Materials and method

A hospital based cross sectional analytical study was done from March 2013 to February 2014. This study was conducted in the Department of Clinical pathology, Department of Neonatology and in the Department of Microbiology and Immunology, BSMMU, Dhaka. The research protocol was approved by the Institutional Review Board of BSMMU, Dhaka. Non probability convenience sampling was used. Total 60 neonates were included in the study. There were 40 cases clinically diagnosed as neonatal sepsis and 20 control neonates with no symptoms or signs of infection were included in this study. Neonates with gross congenital anomalies, chromosomal abnormalities and with severe jaundice due to blood group (ABO, Rh) incompatibilities were excluded from this study. After taking inform written consent from patient's attendant, blood sample were obtained from peripheral venipuncture in all neonates within 24 hours of admission with all aseptic precaution. A total 3.5 ml venous blood was taken of which 1.5 ml was collected in EDTA tube for complete blood count, PBF and for neutrophil CD64 estimation and another 2.0 ml for blood culture. Complete blood count was estimated by automated hematology analyzer (Sysmex XT-4000i), which again rechecked manually by peripheral blood film in Clinical pathology Department. Neutrophil CD64 was measured by BD FACS verse flow cytometer and blood cultures were done by Bactec microbial detection system (Becton Dickinson - 9240) in the Department of Microbiology and Immunology, BSMMU. Cut off value of neutrophil CD64 was (>10%). P value reached from chi square test and fisher exact test. Receiver-operator characteristic (ROC) curve of neutrophil CD64 for prediction of neonatal sepsis was done. All statistical computations were performed by using SPSS (17.0).

Test procedure of Neutrophil CD64

100µL of whole blood was taken in a FACS tube. Added 20 µL of CD64 antibody/antibody cocktail (CD45 & CD64) on vortex. Incubated in dark at room temperature for 10-15 minutes. Then added 2 ml FACS lyses solution on vortex. Incubated in dark at room temperature for 10-12 minutes. Spin cell at 200-300 g for 5 minutes. Aspirate/discard supernant. Wash cell once/twice with sheet fluids. Add 2ml of sheet fluid on vortex, spin at 200-300 g for 5 minutes. Then discard supernant. Resuspend cell in 0.5 ml of sheet fluid. Run on BD FACS verse flow cytometer. Flow cytometric analysis was performed to collect log green fluorescence, log right-angle light scatter, and forward light scatter signals using a Cytoron absolute flow cytometer.

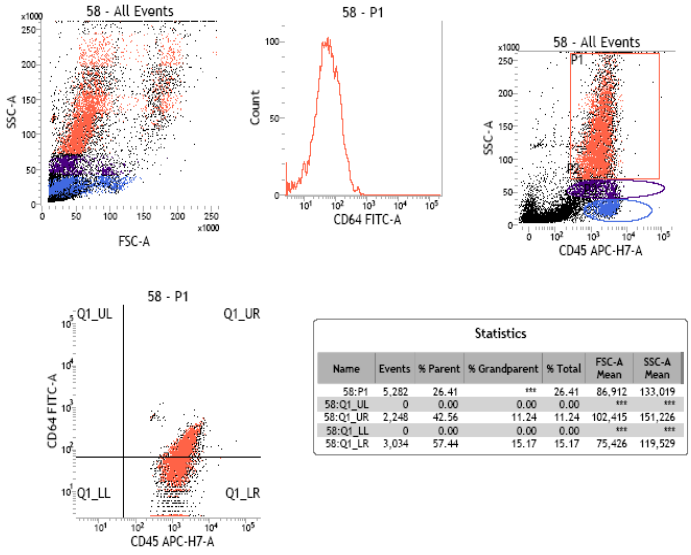

Fig: Flowcytometric analysis of neutrophil CD64 in neonates with sepsis.

Results and observations

This study included 60 patients with a mean age of 5.9±6.49 days. Table I shows demographic status of the study population. Age group, 0-3 days were 25(62.5%) in cases and 05(25.0%) in control group and age group >3 days 15(37.5%) were in cases and 15(75.0%) in control group ($p<0.05$). Male were predominant ($p>0.05$). Gestational age, preterm were more 33(82.5%) in cases than 08(40%) in control group. Birth weight, VLBW & LBW were 31(77.5%) in cases and 04(20%) in control group ($p<0.05$) and normal weight 9(22.5%) were in cases and 16(80%) in control group. Premature rupture of membrane were 30(75%) in cases group & none in control group ($p<0.05$). Chi-square test showed there were significant difference in gestational age, birth weight and premature rupture of membrane among the two groups of the study population ($p<0.05$).

Table II shows mean difference between cases and control neonates with laboratory finding of neutrophil CD64. Mean neutrophil CD64 were 36.03 ±25.70 in cases and 4.85 ±2.95 in control group ($p<0.05$). Values (mean SD) were expressed.

In this study, out of 40 clinically diagnosed neonatal sepsis, blood culture was found positive in 9 (22.5%) cases. (Figure 1)

Percentage of expression of neutrophil CD64 was higher in culture positive sepsis (77.07±15.07) than culture negative sepsis (26.56±13.46) (Table III). The difference was statistically highly significant ($P<0.001$) between culture proven and unproven sepsis with neutrophil CD64.

Positive (>10%) Neutrophil CD64 was found in 32(80.0%) with clinically diagnosed neonatal sepsis and negative (≤10%) neutrophil CD64 was found in 08(20.0%) with neonatal sepsis. Positive (>10%) was neutrophil CD64 found in

none in control group and negative (≤10%) neutrophil CD64 was found in 20(100.0%) in control group (Table IV). The difference was statistically significant between two groups ($P<0.05$).

Sensitivity of neutrophil CD64 was 100%, specificity 54.9%, accuracy 61.67%, positive and negative predictive values were 28.13% and 100% respectively.

Receiver-operator characteristic (ROC) were constructed using neutrophil CD64 level and blood culture result of the neonates with sepsis. It gave neutrophil CD64 level cut off value (>10%) as the value with a best combination of sensitivity 100%, specificity 54.9% and area under the ROC curve for neutrophil CD64 0.970. Area under the ROC curve for blood culture 0.613 with sensitivity 22.5% and best specificity 100% (Figure 2).

Discussion

Diagnosis of neonatal sepsis is still a challenge, as there is no single reliable test for early diagnosis. Currently blood culture is the most reliable method for detection of bacterial infections. But the sensitivity of blood culture is low, longer time required for report and false negative result may be found. Culture positive sepsis is a small proportion of a larger group of clinical sepsis (with negative blood cultures). So it is clear that to manage neonates with sepsis properly, a single reliable marker of infection is needed, to avoid unnecessary antibiotic therapy. In this study, we tried to determine the neutrophil CD64 expression as an immunological marker for rapid diagnosis of neonatal sepsis.

This study included 60 patients with 40 clinically diagnosed sepsis neonates and 20 control neonates who did not have any symptom or sign of sepsis. In sepsis group, early onset was observed more (62.5%) than that of late onset of sepsis

(37.5%). This observation is consistent with the findings of others in BSMMU [9,18]. Among the infected newborns, male were predominant (57.5%). It is due to the factors regulating the synthesis of a globulin situated on the X chromosome. Another fact is that male is more privileged in our society**.** Predominance of male babies suffering from neonatal sepsis was found in other study[9,18,19].

Preterm (82.5%) and low birth weight babies (77.5%) are more susceptible to infection. Higher susceptibility of infection in preterm and low birth weight babies might be due to low level of IgG and lower defense mechanism. There were significant differences in means of gestational age and birth weight between neonates. These findings showed that prevalence of infection in neonates is inversely related to gestational age and birth weight. Duration of premature rupture of membrane (PROM) for >24 hours has to be an important risk factor in neonatal septicemia because PROM poses of ascending infection to the fetus. In our study, PROM was 75% in septic neonates and none in control group. This finding is consistent with the study of others[9].

In this study, out of 40 clinically diagnosed neonatal sepsis, blood culture was found positive in 9 (22.5%) cases. Khaleda et al., (2010) in BSMMU, found 12% neonates as culture positive sepsis. This may be due to faulty sterile technique in collection procedure, insufficient sample volumes, intermittent or low-density bacteraemia, or suppression of bacterial growth by earlier antibiotic administration and delayed arrival of patients. In the present study, there was high percentage of expression of CD64 on neutrophils in patients (36.03 ±25.70) when compared with controls (4.85±2.95) and also their percentage of expression was higher in culture positive sepsis (77.07±15.07%) than culture negative sepsis (26.56±13.46). These results are consistent with another study[20]. Our study showed high significant difference (P=<.001)

between patients and controls regarding mean percentage of expression of CD64 on neutrophils. Similar results by Azza et al., (2013); Young et al., (2012); Dhlamini et al., (2011), repented that quantitation of the neutrophil CD64 is a specific indicator of sepsis than the other available diagnostics tests. The present study showed statistically significant difference (P=<.001) between culture proven sepsis and culture negative sepsis regarding percentage of expression of CD64 on neutrophils. Similarly Azza et al., (2013), founded that there was a highly significant difference between patients with positive blood cultures and those with negative blood cultures regarding the percentage of CD64 positive neutrophils. There are many advantages of using neutrophil CD64 expression as an indicator of neonatal sepsis, as the quantitation of neutrophil CD64 is rapid (<60minutes) and only minimal blood volume (100 µl) is used[17](Davis BH., 2006). In the present study, neutrophil CD64 showed high sensitivity 100%, specificity 54.9%, PPV, 28.13% and also high NPV 100%. Specificity and PPV were low because of large number of false positive result. This may be due to small sample size and blood culture was found positive only in 22.5% cases of neonatal sepsis. This findings coincided with the outcome of numerous studies done for the diagnostic performance of neutrophil CD64 in neonatal sepsis in view of the high sensitivity and negative predictive values[1,10,20,23,24]. The results of our study also showed significantly elevated levels of CD64 in septic neonates when compared to healthy controls. The results of present study clearly indicated that measurement of neutrophilCD64 can be useful for diagnosis of neonatal sepsis in early diagnosis of neonatal sepsis.

Conclusion

Flow cytometric assessment of neutrophil CD64 may be considered as a rapid and reliable marker for the diagnosis of bacterial neonatal sepsis in comparison to other conventional and routine diagnostics markers. Further large scale study may be conducted to get more precise result.

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Table I: Demographic characteristics of the study population (n=60)

	Study group				
	Cases n=40		**Control** n=20		**P value**
Age group	**N**	**%**	**N**	**%**	
0-3days	25	62.5	05	25.0	0.006[s]
> 3 days	15	37.5	15	75.0	
Sex					
Male	23	57.5	12	60	1.0[ns]
Female	17	42.5	08	40	
Gestational age					
Preterm <37 wks	33	82.5	08	40	0.002[s]
Term ≥ 37 wks	07	17.5	12	60	
Birth weight					
Very low birth weight (VLBW)≤ 1500 gm	11	27.5	00	00	<0.001[s]
Low birth weight (LBW) >1500-2499 gm	20	50.0	04	20	
Normal weight ≥2500 gm	09	22.5	16	80	
Premature rupture of membrane (PROM)					
Yes	30	75.0	0	00	<0.001[s]
No	10	25.0	20	100	

s= significant ns= non significant

Table II: Laboratory test results of neutrophil CD64 in cases and control (n=60)

Parameters	**Case(n=40)**		**Control(n=20)**		**P value**
	Mean Min-max	±SD	Mean Min-max	±SD	
Neutrophil CD64 (%)	36.03 5.01-96.67	±25.70	4.85 0.61-7.90	±2.95	<0.001 [s]

s= significant ns= non significant

Blood culture is the gold standard for diagnosis of neonatal sepsis. It was done in all cases. Out of 40 cases 9 neonates (22.5%) were blood culture positive and 31(77.5%) culture negative. This study found that most of clinically diagnosed sepsiswere blood culture negative. Statistical analysis revealed that there was significant difference ($p<0.05$).

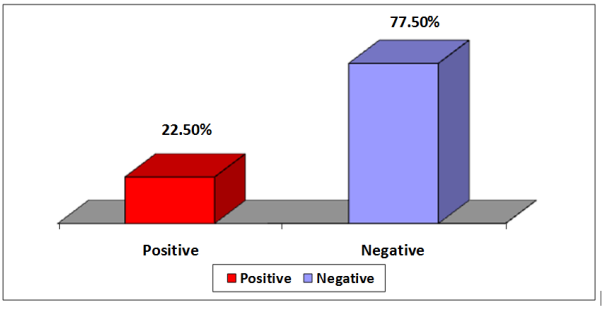

Figure 2: Distribution of blood culture result in septicemic neonates (n=40)

Table III: Results of neutrophil CD64 detected by flow cytometer in culture positive and culture negative cases of neonatal sepsis (n=40)

Parameters	Blood culture		P value
	Positive Mean (±SD)	Negative Mean (±SD)	
Neutrophil CD64	77.07(±15.07)	26.56(±13.46)	<0.001[s]

s= significant

Table IV: Distribution of study population according to neonatal sepsis with Neutrophil CD64 (n=60)

Neutrophil CD64	Study group				P value
	Cases n=40		Control n=20		
	N	%	N	%	
Positive (> 10%)	32	80	00	--	<0.001[s]
Negative (≤10%)	08	20	20	100	
Total	40	100	20	100	

s= significant

P value reached from Fisher's Exact Test

Table V: Validity of different laboratory tests with blood culture (n=60)

	Sensitivity	Specificity	PPV	NPV	Accuracy
Neutrophil CD64	100%	54.9%	28.13%	100%	61.67%

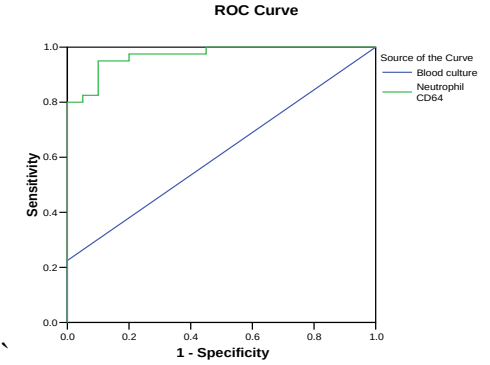

Figure 3: Receiver-operator curves of neutrophil CD64 and Blood culture