Role of CD64 in the Diagnosis of Neonatal Sepsis

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ABSTRACT

Introduction: Early diagnosis of neonatal sepsis is a challenge because of subtle and non specific clinical signs. The gold standard test -blood culture has a sensitivity of only 25-55%. Hence, there is a need to study other markers for diagnosis of neonatal sepsis. CD64, a leukocyte surface antigen, is up regulated during infection and sepsis. Study objective was to evaluate the utility of CD64 expression in diagnosis of neonatal sepsis and to compare it with other diagnostic markers.

Material and Methods: A cross-sectional study was conducted in neonates with clinical sepsis. Full sepsis work up including Total leukocyte count, Absolute neutrophil count, Band count, Immature to total leukocyte ratio, C-reactive protein (CRP), Haematological Scoring System (HSS) and blood culture was carried out. Neonates were divided into Group 1 (Blood culture positive) and Group 2 (Blood culture negative). A group of healthy neonates (group 3) was enrolled as control. Expression of CD64 on neutrophils was measured by quantitative flowcytometry using FITC Mouse Antihuman CD64 monoclonal antibody. Cut-off for CD64 was derived and Qualitative variables assessed.

Results: 32, 92 and 33 neonates were enrolled in Group 1, 2 and 3 respectively. The optimal cut off value for CD64 marker was 37.55 Median Fluorescent Intensity (MFI). After comparing different haematological indices, CD64 ($\geq$ 37.55MFI), CRP ($\geq$10mg/l) and HSS ($\geq$ 3) could discriminate between clinical sepsis and controls ($P \leq 0.05$). CD64 expression showed highest sensitivity (96.77%) and specificity (100%).

Conclusion: CD64 expression is a very promising marker for the diagnosis of neonatal sepsis.

Keywords: CD64, Neonatal Sepsis, Septicaemia, CRP, Flow Cytometry

INTRODUCTION

Sepsis is the most common life threatening diseases among neonates. Despite major advances in management of neonates, neonatal sepsis remains important cause of morbidity and mortality.[1] Early diagnosis of neonatal sepsis is difficult because of subtle and non specific clinical signs, which are indistinguishable from those caused by a variety of non infectious disorders, such as aspiration syndromes, maladaptation, and respiratory distress syndrome.[2] However, early diagnosis and treatment of neonatal sepsis is essential to prevent severe and life threatening complications. It also prevents unnecessary exposure of antimicrobial to newborns and helps in preventing emergence of resistance in bacteria commonly encountered in neonatal sepsis.

The gold standard test in diagnosis of neonatal sepsis is isolation of causative microorganism by blood culture. However, sensitivity of blood culture is 25 -55%[3], results of blood culture are not available until 24-48 hours and often negative in cases of pneumonia and meningitis, or even in fatal generalized bacterial infections.[2] Considering the high mortality rate of neonatal sepsis, a diagnostic marker with a high sensitivity and negative predictive value close to 100% is needed for better management of neonatal sepsis. Hence, there is need to study various other markers for diagnosis of neonatal sepsis.

With increasing understanding of the inflammatory cascade of sepsis and rapid advances in diagnostic technologies, various potential infection markers have been investigated, each of them having their own advantages and limitations.

Various physiological markers, hematological indices[4], acute phase reactants like C- reactive protein (CRP), Procalcitonin[5] and cytokines[5] have been studied to accurately identify neonatal sepsis. Hematological indices have poor specificity for diagnosing sepsis and it can also have subjective errors especially in estimating the band count and its derived immature/total neutrophil ratio.[6] CRP is a late marker of neonatal infection peaking approximately 24 hours after infection.[5,7] It cannot truly determine sepsis as it may be increased in other conditions also. Procalcitonin has a natural fluctuation in the immediate postnatal period, requiring careful adjustment in normal range. Recent studies have shown that sensitivity of Procalcitonin is low at birth, by far the most critical decision point when evaluating a newborn to rule out sepsis.[8] Majority of cytokines have high negative predictive values, but these have not been adopted for general medical use. This is partly attributable to the large amount of blood required, the long interval for cytokine results and cost involved.[6]

Recently, leucocyte cell surface antigens have been reported as a diagnostic marker for neonatal sepsis. These include CD11b, CD64, CD59, CD45RO and CD25. Among these cell surface markers, CD64 had the highest sensitivity and specificity at the onset and upto 24 hours after the initial clinical presentation for diagnosing late onset bacterial infection and Necrotising Enterocolitis.[7] CD64, a leukocyte surface antigen, is expressed at low concentration on

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surfaces of non-activated neutrophils. CD64 is a high affinity Fc receptor which is up regulated during infection and sepsis. Flowcytometry has made it possible to quantitate neutrophil CD64 expression rapidly, with precision and more importantly for neonates, with minimal blood volume.[6] CD64 expression in bacterial infection in preterm and term neonates is similar in magnitude compared to older children and adults[9]. If studied in depth, CD64 expression may help in discontinuing antibiotic treatment early without waiting for definitive microbiologic culture results. Our aim of the study was to evaluate the utility of CD 64 expression in diagnosing neonatal septicemia in comparison to other diagnostic markers for neonatal sepsis.

MATERIAL AND METHODS

This cross sectional study was conducted in a tertiary care, multi-speciality, teaching hospital, Mumbai over a period of one year after taking Institutional review board permission (EC/155/2011).

All neonates diagnosed as clinical sepsis by the Neonatologist according to the definition given by National Neonatology Forum's[10] for hospitals were considered for inclusion in the study. Neonates already on antimicrobial therapy were excluded from the study.

Consecutive neonates with a clinical diagnosis of neonatal sepsis, admitted to neonatal intensive care unit, were enrolled after obtaining written informed consent from their parents or legal guardians.

After obtaining results of blood culture they were divided into two groups.

Group 1: Culture positive neonatal sepsis (clinical+ve, culture+ve)

Group 2: Culture negative neonatal sepsis (clinical+ve, culture-ve)

A separate group of healthy neonates (group 3) was enrolled as control group. All the three groups were age matched. Minimum of 30 neonates were included in each group.

A full sepsis work up of all neonates was carried out, at the time of hospitalization of neonates before starting any antimicrobial therapy. An additional 0.3-0.4 ml of blood was collected at 0 hours for determining CD64 expression in an EDTA vacutainer / microtainer for cases and controls. 0 hours is defined as the time of first blood collection for full sepsis evaluation.

A repeat blood specimen (0.3-0.4 ml) of neonates with clinical sepsis was also collected after 72 hours after that for determining CD64 expression.

Full sepsis screen included Total leukocyte count (TLC), Absolute Neutrophil Count (ANC), Band count, Immature to Total leukocyte (I/T) ratio, C- reactive protein (CRP) as per standard protocol. Along with sepsis screen, blood culture was also performed using BACTEC TM 9050 Blood Culture System (Becton Dickinson and Company, Sparks, MD, USA). For evaluation of neonatal sepsis Hematological scoring system (HSS) was also taken into consideration.[11]

Expression of CD64 on neutrophils was measured by quantitative flowcytometry with a FACS Calibur™ flowcytometer (Becton Dickinson Immunocytometry Systems,San Jose, California, U.S.A) using FITC Mouse Antihuman CD64 monoclonal antibody. This monoclonal antibody was stored undiluted at 4 °C and protected from prolonged exposure to sunlight. The specimen preparation and flow cytometer set up were based on the manufacturer's instructions (Figure 1) and the specimens were analysed using the Cell Quest Pro computer software (Becton Dickinson Immunocytometry Systems) using the FACSCalibur™ Flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, California, U.S.A).

STATISTICAL ANALYSIS

Cut-off for CD 64 was derived using ROC curve in MedCalc for Windows, version 11.3.3.0 (MedCalc software, Mariakerke, Belgium). Quantitative data was represented using Mean ± SD (Standard Deviation). Analysis of quantitative data between qualitative variables with more

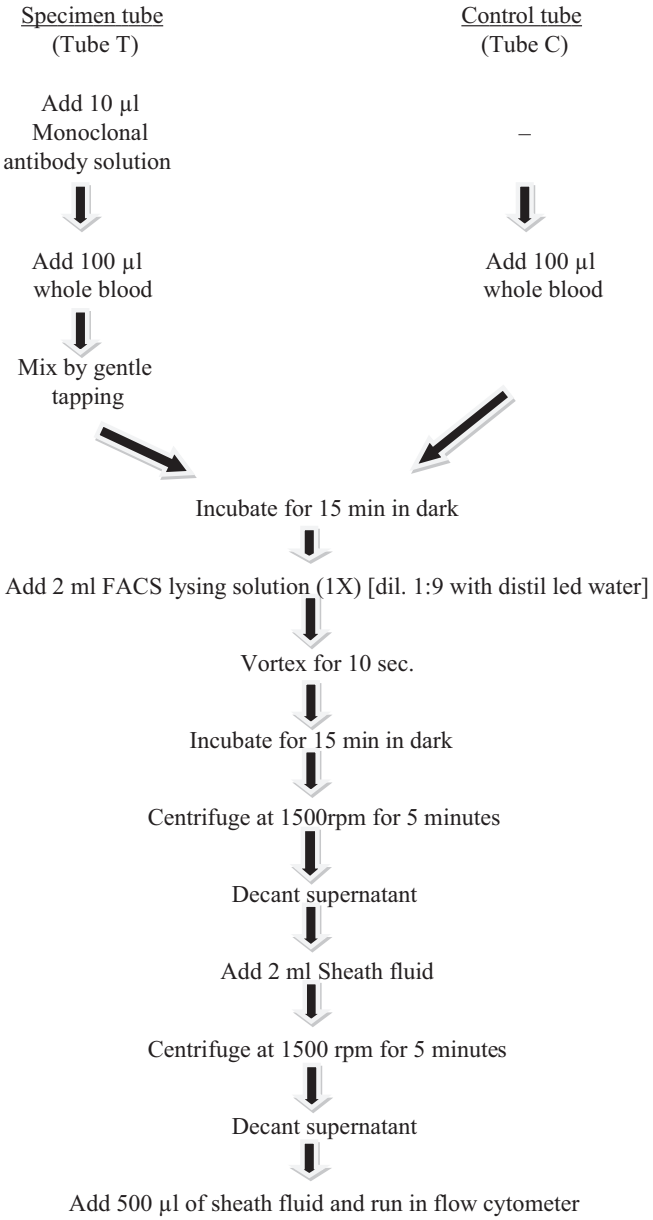

Figure-1: Steps involved in CD64 expression estimation by Flowcytometry

than two sub groups was assessed using One-way ANOVA if the data passed 'Normality test' and by Kruskal-Wallis test if data failed 'Normality test' with application of appropriate Post Hoc test if P-value of ANOVA came statistically significant. Diagnostic efficacy was assessed through Sensitivity, Specificity, PPV, NPV, and Positive Likelihood measurements for diagnosis of Sepsis as gold standard and various measurements like CRP, CD64 and HSS. All statistical tests were performed by SPSS Version 17

RESULTS

124 episodes of suspected clinical neonatal sepsis have been investigated. 32 of the 124 episodes were identified as culture positive, 92 as culture negative episodes after performing BACTEC™ 9050 blood culture system (Becton-Dickinson, Sparks, MD). 33 healthy neonates were included as the control group.

As there is no recommended diagnostic cut off value for any of the cell surface antigens in neonatal sepsis, Receiver operating characteristic (ROC) curve was constructed for each sampling time point for CD64 expressions at 0 hours. The ROC graph with maximum area under the curve as 0.998 and at 95% confidence interval was chosen and the optimal cut off value for CD64 marker was determined as 37.55 Median Fluorescent Intensity (MFI) (Figure 2). The sensitivity and specificity of the test is 96.77% (95% CI - 91.9% - 99.1%) and 100% (95% CI - 89.4% - 100%) respectively for correctly identifying cases of sepsis. There was no significant difference in expression of CD64 at 0 hours and after 72 hours of onset of sepsis (P= 0.121). CD64 expression was noted to be markedly raised both at 0 hours and 72 hours of onset of clinical neonatal sepsis.

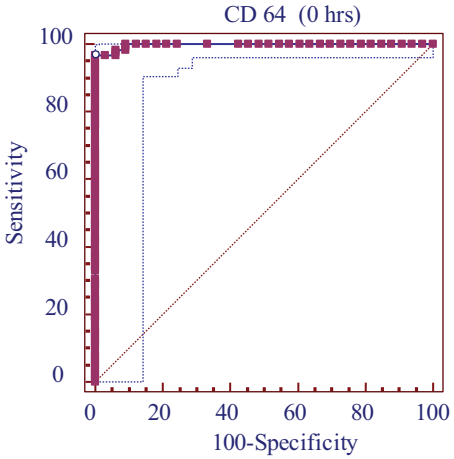

Figure-2: Receiver operating characteristics curve (ROC) curve for CD64 expression

Mean with standard deviation of CD64, CRP, TLC, ANC, Band count, I/T ratio, Platelet count and HSS for all three neonatal groups are presented in Table 1. After comparing all above mentioned haematological indices for identification of neonatal sepsis CD64 ≥ 37.55 MFI (P = 0.0001), CRP≥10mg/l (P = 0.0001), HSS ≥ 3 (P = 0.0001) TLC (P = 0.00945) and ANC (P = 0.00196) were found to be significantly different between the three groups. However on performing all pair wise multiple comparison procedures (Dunn's method) it was found that only CD64, CRP and HSS could discriminate between clinical sepsis (both between culture positive and culture negative neonates) and controls (P ≤ 0.05).

In addition, a comparison of the diagnostic utilities of the three best markers CD64, CRP and HSS in combination versus individual markers, suggested that CD64 expression (≥37.55 MFI) showed highest sensitivity (96.77%) and specificity (100%) for diagnosing sepsis cases. Combining it with other markers of neonatal sepsis did not improve its sensitivity or specificity (Table 2).

DISCUSSION

Early diagnosis of neonatal sepsis is essential to prevent severe and life threatening complications. However, Laboratory confirmation of neonatal sepsis is usually a problem as blood culture report takes a long time. Hence, we require a test which can be used for the early diagnosis of neonatal sepsis as there is also a possibility of having clinical neonatal sepsis with negative blood culture report. For the diagnosis of early onset sepsis in clinical practice, the sensitivity of any test is more important compared to the specificity, as immediate management is recommended to reduce morbidity and mortality.

Parameter	Sepsis Group		Control Group 3 (n=33) Mean (±SD)	P value	Dunns method (P value)		
	Culture positive Group 1 (n=32) Mean (±SD)	Culture negative Group 2 (n=92) Mean ±SD)			Grp 1 vs. Grp 2	Grp 1 vs. Grp 3	Grp 2 vs. Grp 3
CD64 (0 hours)	73.14 (±29.82)	63.81 (±25.48)	19.94 (±7.10)	0.0001	>0.05	≤ 0.05	≤ 0.05
CRP	10.72 (±15.48)	5.69 (±14.15)	1.18 (±1.70)	0.0001	≤ 0.05	≤ 0.05	≤ 0.05
TLC	16923.81 (±7311.75)	14115.24 (±5276.39)	14453.18 (±11314.60)	0.00945	>0.05	≤ 0.05	>0.05
ANC	12001.63 (±5763.99)	9265.27 (±4725.06)	8241.91 (±4813.15)	0.00196	≤ 0.05	≤ 0.05	>0.05
Band count	2.44 (±3.01)	2.47 (±2.33)	1.52 (±1.44)	0.108	-	-	-
I/T ratio	0.06 (±0.04)	0.05 (±0.03)	0.04 (±0.01)	0.121	-	-	-
Platelet count (lakhs)	1.62 (±0.80)	1.61 (±0.69)	1.74 (±0.36)	0.325	-	-	-
HSS	2.97 (±0.47)	2.62 (±0.68)	1.48 (±0.67)	0.001	>0.05	≤ 0.05	≤ 0.05

MFI = Median fluorescent Intensity, CRP = C- reactive protein, TLC = Total leucocyte count, ANC = Absolute neutrophil count, I/T ratio= Immature to total leucocyte ratio and HSS = Hematological scoring system, Grp= Group

Table-1: Comparison of various haematological indices in the different groups

CD64 has already been identified as a high affinity Fc- gamma receptor of IgG antibody involved in the process of phagocytosis and intracellular killing of opsonised microbes.[12] It has several characteristics that make it well suited for clinical application. Flow cytometric analysis has the advantage over conventional immunological assay methods for being able to localize the activated markers to a specific cell type. Expression of the CD64 antigen on neutrophils has been under investigation for some years as a biomarker of infection and sepsis.[13,14] On resting neutrophils, CD64 expression is low and after activation it is significantly up regulated within few hours. Also, in the current study cut off value for CD64 expression was calculated as 37.55 MFI with high sensitivity (96.77%) and specificity (100%) and its expression was high both at the time of sepsis evaluation, and at 72 hours after sepsis evaluation even though empiric antimicrobial treatment was started. Hence, this test can be used for detection of sepsis from 0 hours to 72 hours**.** Moreover, CD64 is relatively stable after blood collection and the assay is straightforward and requires only small specimen volume.

Currently used parameters for diagnosis of neonatal sepsis, such as TLC, ANC, band count, I/T ratio, platelet count, HSS and C reactive protein were compared with CD64 expression and evaluated for their utility in diagnosis of neonatal sepsis.

Total leukocyte count (TLC) is of little clinical use in the diagnosis of neonatal infection because of wide variation in values. I/T ratio, band count and platelet count were also not consistent and not found to be significantly associated with neonatal sepsis. As no single individual haematological parameter is superior in comparison to another in predicting neonatal sepsis, a combination of these parameters in the form of haematological scoring system (HSS) has been recommended.[11] In current study haematological scoring system with a cut off value as 3, had sensitivity of 74.19%, but disappointingly low negative predictive value of 50%. Also, this scoring system is not widely adopted because of its unfavourable diagnostic values, complexity of the scoring method, and the fact that some of the tests are labour intensive and require a highly trained technician to produce an accurate result. Low platelet counts and morphological changes in neutrophils are also often severe and late signs of infection.

In the present study, CRP had a very low sensitivity (12.10%), but good specificity (100%) this may be because CRP estimation was done at 0 hours and it is known that concentrations of CRP increase at around 24 hours after onset of infection.[15] During the neonatal period, an established upper normal CRP level of 10 mg/L has been identified in many studies[16,17] but still, the CRP diagnostic accuracy varies widely within an unacceptable range of sensitivity.[18,19] This may be related to the arbitrary choice of optimal cut off points.[18,19]

In the present study, though CRP estimation showed statistically significant difference between the three groups, mean value of CRP estimation was 5.69 in culture negative patients which is lesser than its cut off value of 10 mg/l. Hence, CRP is not a good indicator to identify neonatal sepsis especially in culture negative group.

With cut off value for CD64 estimation as 37.55 median fluorescence intensities, sensitivity and specificity of this test for the diagnosis of neonatal sepsis was found to be 96.77% and 100% respectively. Even if CD64 expression was combined with other diagnostic parameters, though the sensitivity of this combination increased marginally, it was at the cost of specificity. Ng PC et al (2004), has also showed that additional use of CRP did not significantly improve the sensitivity

Diagnostic Parameters	CD64(0hr) ≥37.55MFI (±95%CI)	CRP > 10 mg/l (±95%CI)	HSS ≥ 3 (±95%CI)	CD64 and CRP (±95%CI)	CD64 and HSS (±95%CI)	CD64,CRP and HSS (±95% CI)
Sensitivity	96.77% (91.93-99.11%)	12.10% (6.93– 19.15%)	74.19% (65.56– 81.61%)	96.77% (91.93– 99.11%)	98.39% (94.30– 99.80%)	98.39% (94.30– 99.80%)
Specificity	100% (89.43-100%)	100% (89.43 – 100%)	96.97% (84.23 – 99.92%)	100% (89.43 – 100%)	96.97% (84.23 – 99.92%)	96.97% (84.23 – 99.92%)
PPV	100% (96.97 – 100%)	100% (78.19 – 100%)	98.92% (94.16 – 99.97%)	100% (96.97 – 100%)	99.19% (95.55 – 99.98%)	99.19% (95.55 – 99.98%)
NPV	89.19% 74.61-96.97%)	23.24% (16.56 – 31.02%)	50% (37.20 – 62.80%)	89.19% (74.61 – 96.97%)	94.12% (80.23 – 99.28%)	94.12% (80.32 – 99.28%)

Table-2: Comparison between CD64, CRP and HSS

PARAMETERS	CD 64	CRP	HSS
Cost per test	Rs 200/-	Rs 150 - 200/-	Rs 230/-
Amount of blood/serum Required	0.4 ml whole blood	0.5 ml serum (1-2 ml whole blood)	2-3 ml blood
Time to result	45 minutes	1 hour	2-3 hours
Methodology	Flow cytometry	Nephelometry / Turbidimetry	Electronic counter (coulter method) plus peripheral smear examination
Technical expertise	Required for processing	Not required	Required for calculating HSS
Single/Batch processing	Single	Preferably Batch	Single

Table-3: Logistics of performing CD64, CRP and HSS.

of neutrophil CD64, but adversely affected the specificity of CD64 expression.[20]

When the technical and financial parameters of various diagnostic modalities were compared the cost per test of CD64 estimation was comparable to both CRP and HSS (Table 3). Also, the requirement of whole blood for the CD64 expression was low as compared to the other two which is particularly advantageous in the case of neonates. The turnaround time (TAT) is also less and specimens can be processed singly. In case of HSS, even though the sample can be processed individually, the TAT is higher and for CRP, it is preferable to run specimens in batches.

CD64 expression test needs technical expertise for processing the specimens and involve one time investment in the purchase of a flow cytometer. Also, there are no recommended measuring parameters and cut off values for CD64 expression measurement. Various authors have used different measuring parameters (CD64 index, phycoerythrin molecules bound / cells, mean fluorescent intensities, median fluorescent intensities) for CD64 measurement and have derived their own cut off values.[2,6,7,9,14]

CONCLUSION

To conclude, CD64 expression measurement is a very promising marker for the diagnosis of neonatal sepsis. It has the added advantage of requiring low blood volumes which is always an advantage in neonates. However, for this test to become commercially available, we need to establish cut off values and use uniform CD64 expression parameter.

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