Diagnostic utility of neutrophil CD64 as a marker of early-onset sepsis in very low birth weight neonates

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ABSTRACT

Background: Sepsis is a major cause of neonatal morbidity and mortality. It is extremely important to make an early diagnosis of sepsis, as early recognition and prompt institution of treatment is critical for improved outcome. **Objectives:** This prospective observational study was aimed to evaluate the diagnostic utility of neutrophil CD64 expression for the identification of early-onset sepsis in very low birth weight (VLBW) neonates. **Methods:** All VLBW neonates with signs and symptoms of sepsis before 72 h of age, born to mother with or without risk factors for sepsis were enrolled. Complete blood cells count, C-reactive protein, absolute neutrophil count, I/T ratio, blood culture, and neutrophil CD64 assessment were performed. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of neutrophil CD64 and its combination with other hematological makers for sepsis were measured. **Results:** A total of 302 VLBW neonates were enrolled; of which, 151 were cases and 151 were controls. Neutrophil CD64 was significantly increased in cases than in controls ($p<0.001$). The highest performance of CD64 was at 0–24 h of age with cutoff value >2.2. Using this cutoff value, sensitivity, specificity, PPV, and NPV of neutrophil CD64 counts were 94.04%, 100%, 100%, and 94.37%, respectively. The combination of CD64 counts with predefined leukocyte count criteria (total leukocyte count <5000) yielded the highest sensitivity, highest NPV, and highest area under curve. Cutoff value for neutrophil CD64 expression in definite sepsis group (blood culture positive) was >3.2 for 0–24 h of age and had sensitivity, specificity, PPV, and NPV of 93.33%, 65.29%, 40%, and 97.53%, respectively. Conclusion: Neutrophil CD64 is a highly sensitive biomarker for diagnosis of early-onset sepsis in neonates.

Key words: *Neutrophil CD64, Receiver operating characteristic curve, Sensitivity, Sepsis, Specificity*

In neonates, sepsis continues to be a major cause of morbidity and mortality [1]. As per the national neonatal-perinatal database (2002–2003), the incidence of neonatal sepsis is 30/1000 live births [2]. The incidence is higher among very low birth weight (VLBW) infants. Early recognition and diagnosis of neonatal sepsis is difficult because of its variable and non-specific clinical presentation. It is extremely important to make an early diagnosis of sepsis because prompt institution of antimicrobial therapy improves outcomes and delayed or missed treatment can have serious consequences. Attempts have been made to seek an ideal early marker of neonatal sepsis. Physiologic parameters, hematologic indices, and cytokine profile have been identifying neonates with sepsis [3-5].

The complete blood count and leukocyte differential assays have relatively poor specificity for diagnosing sepsis. The band cells and leftward shift of myeloid immaturity measurements may improve diagnostic yield, but their subjective measurement is problematic. The gold standard for the diagnosis of neonatal sepsis is blood culture, but it has some limitations. There may be delay in the final culture results for 48–72 h. This results in admission to a NICU and separation from parents as well as increased cost to the health-care system. Genuine bacteremia may remain undetected in a significant proportion of infected cases [6]. Therefore, the need persists for improved diagnostic indicators of neonatal sepsis.

The CD64 is expressed at very low concentration on surface of neutrophils in the absence of bacterial infection. During bacterial sepsis, the CD64 expression increases [6]. CD64 expression on neutrophil is induced by immunoglobulin G (IgG), interferon gamma, or granulocyte colony-stimulating factors [7]. Following contact with an antigen, the antigen-IgG complex binds to CD64. This process further increases CD64 expression. Available literature shows that neutrophil CD64 seems to be a highly sensitive and specific biomarker for sepsis. However, nearly, all studies included relatively small patient numbers [8]. There is an enormous variation in criteria for selecting patients, differences in the definition of sepsis, as well as poorly standardized analytical methodology. Moreover, there is a paucity of data in this age group and very few studies have been conducted on this topic, so far none of which are carried out in India.

The present study is an effort to fulfill the gap in the existing literature. This study was planned to evaluate the efficacy of

neutrophil CD64 expression for diagnosis of early-onset sepsis (EOS) in VLBW neonates. We also planned to compare neutrophil CD64 marker with other currently used infection markers including total leukocyte count (TLC), absolute neutrophil count (ANC), C-reactive protein (CRP), platelet counts, and blood culture.

METHODS

This prospective observational study was conducted at a tertiary care teaching institute from Delhi, India, from January 2015 to December 2015. The study was approved by the ethical committee. Written informed consent was obtained from parents or legal guardian before enrollment. Study population was VLBW neonates admitted in neonatal division of the department of pediatrics. Inclusion criteria were neonates with birth weight <1500 g and postnatal age ≤72 h, admitted to NICU with signs and symptoms of sepsis such as hyperthermia or hypothermia, tachycardia or bradycardia, CRT >3 s, severe chest indrawing, abdominal distention, bulging fontanel, decreased movements, feeding difficulty, convulsion, umbilical sepsis, and multiple skin pustules and born to mothers with or without risk factor for sepsis. Neonates with chromosomal or gross congenital abnormalities were excluded from the study.

Cases were all VLBW neonates with culture-positive sepsis or clinical sepsis as per the CDC definition. This included neonates having all of the following criteria: Existence of predisposing risk factors (maternal fever within 7 days before delivery or foul-smelling liquor or prolonged rupture of membranes [>18 h]) or radiological evidence of pneumonia or culture from any body fluid comes positive or positive sepsis screen. Controls were all VLBW neonates initially suspected of having sepsis but have negative blood culture and have negative sepsis screen. Assuming dropout rate of 10% and with alpha=0.05 and p=80%, the study was carried out on 151 cases and equal number of controls.

For all these neonates, venous blood sample was drawn under aseptic precaution for blood culture, sepsis screen, and CD64 within first 24 h of birth. A second blood sample was obtained between 25 and 72 h after birth from those neonates who required additional laboratory tests. 10 ml of whole blood was incubated with fluorescein isothiocyanate (FITC) conjugated monoclonal antibody against CD64 (anti-CD64) for 10 minutes. This was followed by red cell lysis by adding lysis reagent. Later, flow cytometry was done using Guava Easycyte flow cytometer to collect log FITC fluorescence, log right angle side scatter, and forward scatter on a minimum of 50,000 leukocytes. Data analysis was performed using light scatter gating to define the neutrophil population, and CD64 positivity was quantified as mean equivalent to soluble fluorescence units using the Guava Easycyte software with a correction for non-specific antibody binding by subtracting values for the isotype control. Interassay standardization and CD64 quantification were performed using FITC calibration beads.

Categorical variables were presented in number and percentage, and continuous variables were presented as mean ± standard deviation. Normality of data was tested by Kolmogorov–Smirnov test. If the normality was rejected, then non-parametric test was used. Quantitative variables were compared using unpaired *t*-test/Mann–Whitney test. Qualitative variables were correlated using Chi-square test/Fisher's exact test. Receiver operating characteristic (ROC) curve was used to find out cutoff point of CD64. Diagnostic test was used to find out sensitivity, specificity, negative predictive value (NPV), and positive predictive value (PPV) of various parameters for the prediction of sepsis. $p<0.05$ was considered statistically significant. The data were entered into MS Excel spreadsheet, and analysis was done using SPSS version 21.

RESULTS

During the study period, 302 VLBW neonates satisfied the inclusion criteria and were included in the study. A total of 413 neutrophil CD64 detections (302 and 111 detections obtained at 0–24 h and at 25–72 h after birth, respectively) were done. Among these 302 VLBW neonates, 151 were belonged to sepsis group (30 - culture proven and 121 - culture negative sepsis) and 151 were belonged to the control group. The demographic data of two groups are summarized in Table 1. Cases were of lesser gestational age than those of control ($p<0.002$). Birth weight was also significantly lower in cases than control ($p<0.003$). Maternal risk factors for sepsis were higher among cases than control ($p<0.011$). However, there was no difference in sex ratio among cases and controls.

The level of hematological markers for sepsis and neutrophil CD64 is summarized in Table 2. The level of CD64 was higher among cases ($p<0.0001$), TLC and ANC were significantly lower in cases ($p<0.0001$), while I/T ratio and CRP were higher in cases in comparison to control ($p<0.0001$). There was no significant

Table 1: Demographic profile and maternal risk factors for sepsis

Variables	Cases (n=151)	Controls (n=151)	p
Gestational age in weeks (mean±SD)	31.82±2.52	32.75±1.92	0.002
Birth weight in grams (mean±SD)	1171.62±161.5	1235.3±136.03	0.003
Sex (Male: female)	76:75	77:74	0.908
Maternal risk factors for sepsis (present)	78 (51.66%)	56 (37.09%)	0.011

SD: Standard deviation

Table 2: Distribution profile of neutrophil CD64 expression in cases and controls

Variable	Study group		p
	Cases, n (%)	Controls, n (%)	
CD64 (0–24 h), (n=302)			
<2.2	9 (5.96)	151 (100.00)	<0.0001
>2.2	142 (94.04)	0 (0.00)	
CD64 (25–72 h), (n=111)			
<3.2	0 (0.00)	46 (41.44)	<0.0001
>3.2	64 (100.00)	1 (2.13)	

Table 3: Comparison of neutrophil CD64 expression with other currently used sepsis markers in the study group

Variable	Number	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	AUC
CD64 (0–24 h) (>2.2)	302	94.04	100.00	100.00	94.37	0.970
CD64 (25–72 h) (>3.2)	111	100.00	97.87	98.46	100.00	0.989
TLC (<5000)	302	68.87	87.42	84.55	73.74	0.781
ANC (<1500)	302	55.63	100.00	100.00	69.27	0.778
I: T (>0.2)	302	49.01	100.00	100.00	66.23	0.745
Platelet count (<1 Lac)	302	11.26	90.07	53.13	50.37	0.507

AUC: Area under curve

Table 4: Sensitivity, specificity, PPV, and NPV and AUC for CD64 expression at 0–24 h and 25–72 h in combination with other parameters of sepsis screen

Variable	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	AUC
CD64 (0–24 h) and TLC	64.90	100.00	100.00	74.02	0.825
CD64 (0–24 h) and I: T ratio	45.03	100.00	100.00	64.53	0.725
CD64 (0–24 h) and ANC	51.66	100.00	100.00	67.41	0.758
CD64 (0–24 h) and platelet count	11.26	100.00	100.00	52.98	0.556
CD64 (0–24 h) and blood culture	19.87	100.00	100.00	55.51	0.599
CD64 (25–72 h) and TLC	64.06	100.00	100.00	67.14	0.820
CD64 (25–72 h) and I: T ratio	51.56	100.00	100.00	60.26	0.758
CD64 (25–72 h) and ANC	46.88	100.00	100.00	58.02	0.734
CD64 (25–72 h) and platelet count	6.25	100.00	100.00	43.93	0.531
CD64 (25–72 h) and blood culture	64.06	100.00	100.00	67.14	0.820

AUC: Area under curve, PPV: Positive predictive value, NPV: Negative predictive value, TLC: Total leukocyte count, ANC: Absolute neutrophil count

Table 5: Distribution of hematological parameters and neutrophil CD64 expression in the sepsis group with respect to blood culture

Variables	Blood culture negative n=121 (%)	Blood culture positive n=30 (%)	p
TLC			
>5000	34 (28.10)	13 (43.33)	0.107
<5000	87 (71.90)	17 (56.67)	
ANC			
<1500	49 (40.50)	18 (60.00)	0.054
<1500	72 (59.50)	12 (40.00)	
I: T ratio			
<0.2	64 (52.89)	13 (43.33)	0.348
>0.2	57 (47.11)	17 (56.67)	
CRP			
Negative	44 (36.36)	13 (43.33)	0.481
Positive	77 (63.64)	17 (56.67)	
CD64 (0–24 h)			
<2.2	9 (7.44)	0 (0.00)	0.206
>2.2	112 (92.56)	30 (100.00)	
CD64 (25–72 h)	n=34 (%)	n=30 (%)	
>3.2	34 (100.00)	30 (100.00)	

ANC: Absolute neutrophil count, TLC: Total leukocyte count, CRP: C-reactive protein

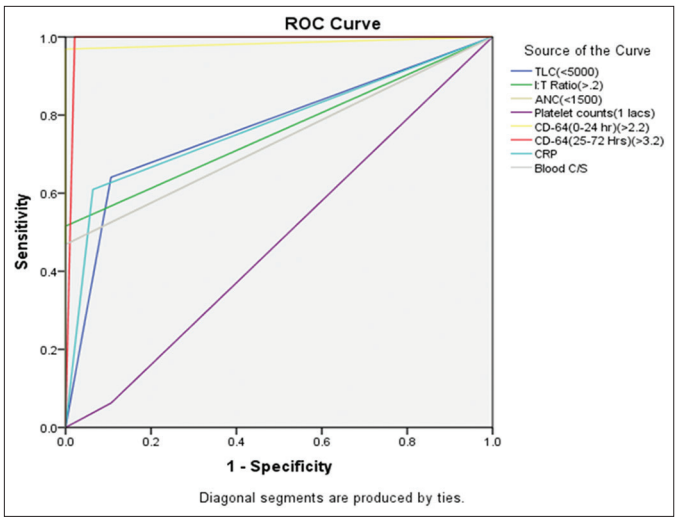

Figure 1: Receiver operating characteristic curve depicting the area under curve for neutrophil CD64 expression and other currently used sepsis markers

difference in platelet count in cases and control. For all parameters, ROC curve was constructed (Fig. 1). The highest area under curve (AUC) was observed using neutrophil CD64 followed by TLC. The neutrophil CD64 has AUC of 0.970 and 0.989 at 24 h and 25–72 h of age, respectively (Table 3). Using a cutoff value of >2.2, the neutrophil CD64 yield a sensitivity, specificity, PPV, and NPV of 94.04%, 100%, 100%, and 94.37%, respectively. Neutrophil CD64 was followed by TLC, ANC, and I/T ratio. Cutoff value for neutrophil CD64 >3.2 at 25–72 h of age yielded sensitivity, specificity, PPV, and NPV of 100%, 97.87%, 98.46%, and 100%, respectively. It was observed that CD64 expression at 0–24 h of age had greater sensitivity, specificity, PPV, NPV, and AUC in comparison to CD64 expression at 25–72 h of age (Table 3).

The combination of CD64 expression (cutoff >2.2 at 24 h of age) with the predefined TLC criteria (<5000) yielded the highest sensitivity of 64.90%, the highest NPV of 74.02%, and also the highest AUC of 0.825 (Table 4). Similarly, the combination

Table 6: ROC characteristics for CD64 expression in blood culture-positive cases

Variables	AUC	Standard error	95% CI	p value	Associated criterion	Sensitivity (%)	Specificity (%)
CD64 (0–24 h)	0.798072	0.0362	0.725122–0.858955	<0.0001	>3.2	93.33	65.29
CD64 (25–72 h)	0.662255	0.0682	0.533166–0.775776	0.0174	>4.4	86.67	44.12

AUC: Area under curve, CI: Confidence interval, ROC: Receiver operating characteristic

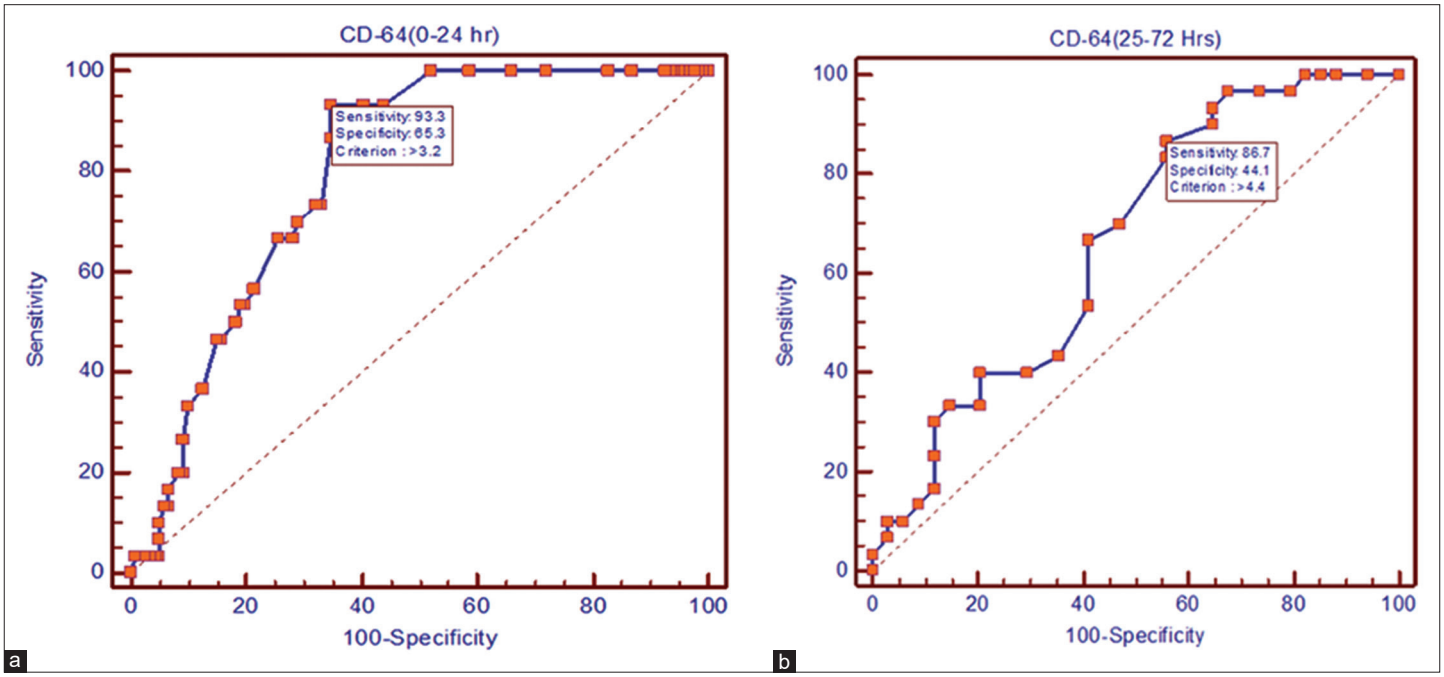

Figure 2: (a) Receiver operating characteristic (ROC) curves for CD64 expression at 0–24 h of age when plotted against blood culture. (b) ROC curves for CD64 expression 25–72 h of age when plotted against blood culture

of CD64 expression (cutoff >3.2 at 25–72 h of age) with TLC yielded sensitivity of 64.06%. NPV 67.14% and AUC of 0.820 which was highest when compared with combination with other hematological parameters. The sensitivity, PPV, and NPV were not calculated for the combination of multiple hematological markers because the basis for defining sepsis in this study was >2 positive hematological indices; this prior definition would produce a positive bias toward higher predictive value. Therefore, only single variable was examined.

There was no statistically significant difference in distribution of sepsis parameter (TLC, CRP, ANC, and I/T ratio) and neutrophil CD64 expression both at 0–24 h and 25–72 h of age in both blood culture-positive and negative group (Table 5). On comparison between CD64 expressions at 0–24 h and at 25–72 h of age in the sepsis group, it was observed that CD64 expression at 0–24 h of age had greater sensitivity, specificity, PPV, NPV, and AUC than the CD64 expression at 25–72 h of age (Table 6 and Fig. 2).

DISCUSSION

In our study, we found that the expression of neutrophil CD64 was significantly increased in the sepsis group as compared to control ($p<0.001$). The results in our study were found to be comparable to the results of previous studies. Soni *et al*. [9] have reported increased expression of neutrophil CD64 in neonates with sepsis. They found that the monocyte/neutrophil CD64 ratio was a highly sensitive marker of culture-positive neonatal sepsis. Ng *et al*. [10] and Livaditi *et al*. [11] have also observed increased expression of neutrophil CD64 in both early- and late-onset neonatal sepsis in VLBW infants. Espinosa *et al.* [12] also demonstrated similar results.

In the present study, an increasing level of neutrophil CD64 expression was observed from birth to 72 h after birth. The highest performance was obtained from 0 to 24 h after birth. These findings are similar to study by Ng *et al.* [13]. Altogether, the results of these studies suggest that a single detection of neutrophil CD64 expression could be reliable diagnostic marker of EOS when performed 0–24 h after birth. This would be particularly important in VLBW neonates where EOS is a com mon condition and if infection could be ruled out at birth, it could significantly affect the therapeutic decision with respect to the antibiotic treatment. Given the high NPV of neutrophil CD64 expression 24 h after the onset of EOS (100.00%), it has the strong potential to influence the initiation, early termination, and duration of treatment by antibiotics.

In the present study, comparison was done between the currently used hematological parameters for the diagnosis of neonatal sepsis and neutrophil CD64 expression in terms of diagnostic tools of evaluation. Neutrophil CD64 expression was found to have highest AUC with the highest sensitivity, specificity, and NPV. This is in agreement with the results demonstrated by Espinosa *et al*. [12], Livaditi *et al*. [11], and

Bhandari V *et al.* [14]. An attempt was made in the present study to see the diagnostic efficacy when combining neutrophil CD64 with currently used hematological markers used for the neonatal sepsis. It was found that neutrophil CD64 in combination with predefined TLC criteria (<5000) yielded the highest sensitivity, NPV, and AUC in comparison with other combinations. Combination of multiple hematological indices was not used because that will result in positive bias toward higher predictive values.

In our study, culture-positive sepsis was found in 30 neonates with sensitivity, specificity, PPV, and NPV of 19.87%, 100%, 100%, and 55.51%, respectively. Using the ROC curve analysis against blood culture, cutoff values for neutrophil CD64 expression were determined as >3.2 at 0–24 h of age and >4.4 at 25–72 h of age. With these cutoffs, the efficacy of neutrophil CD64 expression was determined in culture-positive group, and CD64 expression at 0–24 h of age had better sensitivity and NPV (93.33% and 97.53%, respectively) with AUC of 0.793 as compared to CD64 expression at 25–72 h of age. Previous studies have also reported the similar findings [9,14-18]. Thus, it may be concluded that early postnatal assessment of the neutrophil CD64 expression showed a good performance in predicting EOS and was the most diagnostic measure of sepsis.

CONCLUSION

Neutrophil CD64 can be incorporated as a very useful diagnostic marker for EOS in neonates. Since we got the high NPV for CD64 expression, it has strong potential to influence the initiation, early termination, and duration of antibiotic therapy.

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