Neutrophil CD64 as A Diagnostic Marker in Neonatal Sepsis

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Abstract: Background: Sepsis in neonates hospitalized in the neonatal intensive care unit is a global problem and is a significant contributor to morbidity and mortality. Although treatment of sepsis has evolved in the last decades with newer therapeutic options, little has changed to improve diagnosis or therapeutic monitoring. **Objective:** This case control study aimed to evaluate the diagnostic utilities of neutrophil CD64 (nCD64) expression for the diagnosis of neonatal sepsis. **Subjects and methods:** The study was performed on 41 neonates with evidence of sepsis admitted in the Neonatal Intensive Care Unit (NICU) of Fayoum University Hospitals as a case group and 19 healthy neonates as a control group. Detailed history and meticulous general & systemic examinations were done. Complete blood count, C-reactive protein **(**CRP), blood culture and CD64 index were done simultaneously at time of evaluation. Neutrophil CD64 was analyzed by flow cytometry. **Results:** Neutrophil CD64% showed moderate sensitivity (70.7%) and moderate specificity (73.7%) with cut off value 17.8 in diagnosis of neonatal sepsis. **Conclusion:** There was a significant difference in the percentage of neutrophils expressing CD64 between the case and control groups so nCD64 can be considered a useful marker in diagnosis of neonatal sepsis.
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Key words: neonatal sepsis, neutrophils, CD64, Flow cytometry.

1. Introduction

Neonatal sepsis is defined as systemic inflammatory response syndrome in the presence of or as a result of suspected or proven infection. However, a worldwide agreement on the definition of neonatal sepsis has not been reached **[1]**.

The incidence of neonatal sepsis is approximately 3–40 per 1000 live births, and the mortality rate ranges from 9% to 20%**[2]**.

The prognosis and outcome of neonatal sepsis depend on early diagnosis and efficient antibiotic therapy. However early identification of neonatal sepsis is difficult because of the nonspecific or minimal clinical presentations **[3]**.

CD64 is a glycoprotein, known as Fc gamma receptor-1 (Fc_γ R1) that binds immunoglobulin G (IgG) with high affinity **[4]**.

CD64 is expressed on antigen presenting cells (monocytes, macrophages and dentritic cells), and to a lesser extent on eosinophils, but only to a very low extent on resting neutrophil **[5]**. During neutrophil activation, under the influence of inflammatory cytokines {Interleukin-12, Interferon gamma (INF γ) and Granulocyte colony stimulating factor (G-CSF)}, there is upregulation of neutrophil CD64. Upregulation of CD64 occurs within four to six hours after stimulation with INF γ or G-CSF **[6]**.

2. Patients and methods

Study design; case control study

Study population and sampling:

This case control study had been carried out in the Neonatal Intensive Care Unit of Fayoum University Hospitals between Augest 2016 and October 2017. It included41 neonates with evidence of sepsis according to Griffin Neonatal Sepsis Score (table 1).

Table (1): Griffin Neonatal Sepsis Score.

Parameter	Finding	Points
Lethargy or hypotonia	Absent	0
	Present	1
Temperature instability	Absent	0
	Present	1
Plasma or serum glucose	≤ 180 mg/dL	0
	> 180 mg/dL (hyperglycemia)	1

Parameter	Finding	Points
White blood cell count	< 5,000 per μL	1
	5,000 - 25,000 per μL	0
	> 25,000 per μL	1
Ratio of immature to total neutrophils	≤ 0.2	0
	> 0.2	2
Feeding intolerance	Absent	0
	Present	2
Respiratory status	Normal	0
	Need for ventilatory support with increase in FIO2 25% from baseline	1
	50% or greater increase in apneic episodes over a 24 hour period after being extubated and stable for 3 days	2
	Severe apnea requiring positive pressure ventilation	2

Inclusion Criteria:

Score ≥ 2 on Griffin Neonatal Sepsis Score.

Exclusion Criteria:

1. Neonates with major congenital anomalies.

2. Neonates with score < 2 on Griffin Neonatal Sepsis Score.

Interpretation:

A score ≥ 2 was associated with sepsis. **[7]**

Control group:

19 healthy stable newborns were enrolled in this study as a control group. They were born to healthy mothers with negative medical and obstetric history, Theywere matched for sex, gestational age, weight and mode of delivery. All were free on clinical examination.

Sample size

Sample size was calculated according to Epi Info 2000. A sample size was selected using a special formula based on the global prevalence of disease at a confidence interval of 95% and precision of (2%). The sample increased by 10% to overcome problems related to non-responses and missing data.

Study power:

The power of study was 80%.

All evaluated neonates were subjected to the following:

- Full antenatal and neonatal history.
- Meticulous general & systemic examinations particularly for signs of sepsis as poor activity, hypotonia, delayed capillary refill, feeding intolerance, Down score for respiratory status **[8]**.

Table (2): Evaluation of respiratory distress using Downes' score:

Test	Score		
	0	1	2
Respiratory rate	<60/min	60-80/min	>80/min
Retraction	No	Mild	Severe
Cyanosis	No	Cyanosis relieved by o_2	Cyanosis on o_2
Air entry	Good	Mild decrease	No air entry
Grunting	No	Audible by stethoscope	Audible with ear

Interpretation: score < 4 no respiratory distress, 4-7 respiratory distress and > 7 impending respiratory failure, blood gases are required **[8]**.

- Investigations including:

A. Complete blood count with differential leucocytic count.

B. C-reactive protein assay:-Semiquantitive latex agglutination method was used.

C. Blood culture:-for cases only

The Bactec 9050 fluorescent instrument was used to detect positive blood cultures.

A volume of 2mL blood was added per blood culture bottle using the pediatric sample sized blood culture bottles (Peds Plus). Subcultures of the positive Bactec samples on blood agar, chocolate agar, and MacConkey agar media were done, and identification of isolated organisms was done by colony morphology, microscopic examination and conventional biochemical reactions.

D. Analysis of neutrophil CD64 expression by flowcytometry:

Samples:

100 μL of whole blood collected from peripheral veins of neonates by sterile venipunctures and put in a sterile vacutainer containing K2 EDTA as anticoagulant used for analysis of neutrophil CD64 expression by flowcytometry. Samples were

processed within 24 hours after collection when held at room temperature (18-22ºC) or within 48 hours when refrigerated (2-8ºC).

Equipment:

Fluorescence Activated Cell Sorter (FACS) flow cytometer (Beckman Coulter Epics XL-MCL).

Reagents:

Monoclonal anti CD64 conjugated with fluorescein isothiocyanate (FITC).

Procedure:

1.100 µL of whole blood incubated for 20 minutes at room temperature in the dark with 10 ml of fluorescein isothiocyanate (FITC)-conjugated anti-CD64 monoclonal antibody or isotype control.

2. At the end of the incubation, the tubes were vortexed for a while before adding 500 ml of the lysis solution and continue vortexing for another while.

3-The tubes were incubated for 15 minutes at room temperature in the dark.

4-500 ml of Phosphate buffered saline (PBS) was added to the tubes and incubated for another 10 minutes before analysis.

Flowcytometric analysis:

1. The proper protocol was loaded with 100000 events (cells) selected to pass in front of the argon laser (488 nm) for each case.

2- Control sample was introduced in the machine where the laser scatter was received on both forward scatter detectors and scale to show the cell population in a basic histogram and to adjust autofluorescence region.

3-The sample tube was then introduced and processed in the same way as the control.

4. After 100000 events were counted, the number of cells expressing the surface markers (CD64) will emit fluorescence signals, which will be summated and multiplied then the computer will analyze the data as a colored histogram**.**

Statistical Analysis

- Data were collected and coded to facilitate data manipulation and double entered into Microsoft Access and data analysis was performed using SPSS software version 18 in windows 7.
- Simple descriptive analysis in the form of numbers and percentages for qualitative data, and arithmetic means as central tendency measurement, standard deviations as measure of dispersion for quantitative parametric data, and inferential statistic test:

For quantitative parametric data:

In-depended **student t-Test** used to compare measures of two independent groups of quantitative data

For qualitative data

Chi square test to compare two or more than two qualitative groups.

Sensitivity and specificity test for testing a new test with ROC curve "Receiver Operating Characteristic".

- The **P-value≤ 0.05** was considered the cut-off value for significance.
- The **P-value≤ 0.01** was considered highly significant.

Ethical Consideration:

This study reviewed by the Fayoum Faculty of Medicine Research Ethical Committee. The researcher informed the participants about the objectives of the study, the examination, investigation that did. Alsothe confidentiality of their information and their right not to participate in the study.

3. Results

Table (3): Comparisons of gender and mode of delivery in different study groups.

Variables	**Cases** (n=41)		**Control** (n=19)		p-value	Sig.
	No	**%**	**No**	**%**		
Gender						
Male	25	61%	11	57.9%	0.9	NS
Female	16	39%	8	42.1%		
Mode of delivery						
Vaginal	17	41.5%	10	52.6%	0.6	NS
C.S	24	58.5%	9	47.4%		
Maternal illness						
N0	22	53.7%	19	100%	**<0.001***	**HS**
Yes	19	46.3%	0	0%		

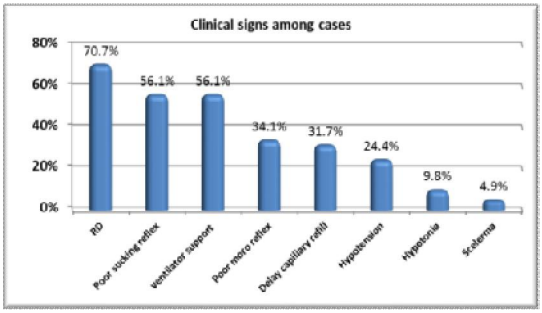


Figure (1): Frequency of clinical signs among cases.

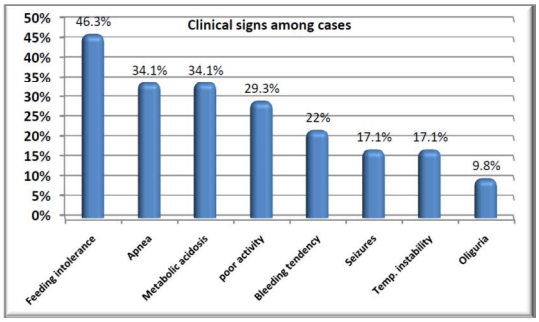


Figure (2): Frequency of clinical symptoms among cases.

Table (4): Frequency of blood culture growth and types of organisms among cases.

Variables	Number (n=41)	%
Blood culture growth		
Negative	29	70.7%
Positive	12	29.3%
Types of blood culture organisms		
E.Coli	1	8.3%
Klebsiella	5	41.7%
Pseudomonas	1	8.3%
MRSA	2	16.7%
Staph.epidermis (CONS)	2	16.7%
Enterobacter	1	8.3%

As regards disease outcome 29.3% of cases died, versus 70.7% discharged from hospital (figure 3).

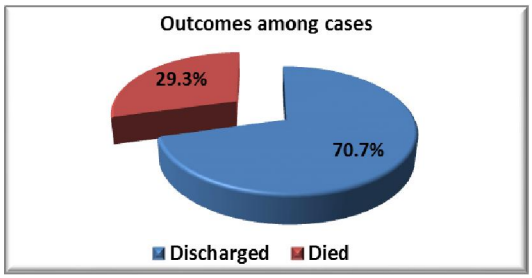


Figure (3): Outcomes among cases.

Table (5): Comparisons of CD 64% in different study groups.

Variables	CD 64%		p-value	Sig.
	Mean	SD		
Cases	26.9	14.1	**0.001***	**HS**
Control	14.9	7.6		

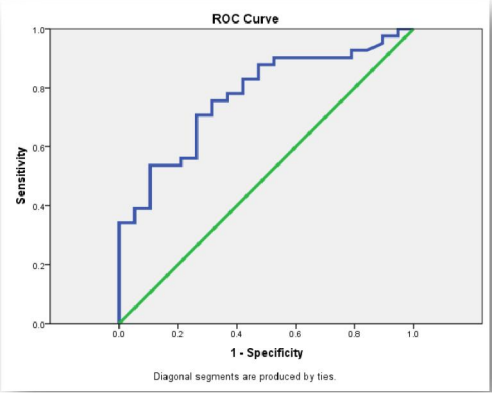
Table (6): Correlation between demographic with other variables among cases.

Variables	Sepsis score	CRP	CD-64
	r (p-value)	r (p-value)	r (p-value)
Weight	-0.56(0.001*)	-0.22(0.2)	-0.37(0.01*)
GA	-0.54(0.001*)	-0.15(0.3)	-0.34(0.02*)
Hospital stay	0.33(0.03*)	0.39(0.01*)	0.3(0.06)

Table (7): Association between Blood culture and other variables among cases.

Variables	Blood culture				P-value	Sig.
	Negative		Positive			
	Mean	SD	Mean	SD		
GA	36.9	3	**34.7**	3.2	**0.04**	**S**
CD-64	22.9	11.8	**36.6**	14.7	**0.003**	**HS**
Hospital stay	17.3	10.9	**24.9**	10.8	**0.04**	**S**
Sex						
Male	19	65.5%	6	50%	0.5	NS
Female	10	34.5%	6	50%		
Mode of delivery						
Vaginal	19	65.5%	7	58.3%	0.2	NS
C.S	10	34.5%	5	41.7%		

ROC curve:



4. Discussion

Neonatal sepsis is considered as one of the major causes of morbidity and mortality for neonates all over the world, particulary in developing countries. **[9]**

Diagnosis of neonatal sepsis is one of the most difficult tasks for physicians and other medical staff due to presence of nonspecific clinical signs and no single reliable test for early diagnosis. **[10]**

In our study, we aimed to evaluate the diagnostic utilities of neutrophil CD64 expression in diagnosis of neonatal sepsis and to define the optimal cutoff value so that it may act as a reference with which future studies can be compared.

In our study, the chief clinical presentations were respiratory distress in addition to poor Moro's reflex and poor suckling. This is in agreement with **Simonsen et al. [11]** who reported that chest manifestations in the form of respiratory distress are the most common as pneumonia is often the presenting infection. On the other hand, **Lim et al. [12]** found that poor activity was the most common presentation.

This study showed statistically significant differences between cases and control groups as regards maternal risk factors as PROM. This finding was consistent with the study of **Paul et al. [10]** who reported PROM for >18 hours has to be an important risk factor in neonatal septicemia because PROM poses ascending infection to the fetus.

In the present study, blood culture was found positive in 29.3% of cases versus 70.7% were negative culture. According to **Shaha et al. [13]** Positive cultures reportedly range from 8-73% in the diagnosis of neonatal 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 this study, Klebsiella was the most commonly organism detected (41.7%) this agree with **Verma et al. [14]** who found that Klebsiella was the most common pathogen (48.21%) in both early and late onset septicemia.

Our results were consistent with **Azza et al. [15]** who found that the majority of sepsis episodes occurred in LBW and premature neonates. Immature host defense mechanisms and invasive life support systems make the premature neonate particularly susceptible to overwhelming infection.

This studyagrees with **Streimish et al. [16]** who found that the rates of infection were similar in males and females. However, this is in disagreement with **Khaleda et al. [17]** who found male Predominance due to the factors regulating the synthesis of a globulin situated on the X chromosome. Male has only one X chromosome, so he is immunologically less protected than the females**.**

As regards the mode of delivery, it was found that the mode of delivery was not significantly associated with increased frequency of sepsis. This agrees with **Elawady et al. [18]** who found that mode of delivery not related to increased incidence of sepsis. On the contrary **Al‐Inany et al [19]** observed that, babies born by vaginal delivery were more likely to have early onset sepsis than those delivered by caesarean section. This may be related to good sterilization and intrapartum chemoprophylaxis which dramatically decreased the risk of sepsis in neonates delivered by caesarean section.

The present study agrees with **Elawady et al. [18]** who found that the long stay in NICU is a major risk factor for neonatal sepsis.

We noticed statistically significant differences between cases and controls as regards CRP which agree with **Dai et al. [20]** who reported that CRP is an excellent marker in diagnosis of neonatal sepsis and has been applied in clinical practice. On the contrary, **Streimish at al. [16]** noted that serial CRP is more helpful for guiding duration of antibiotic therapy, rather than making the diagnosis of sepsis.

We noted statistically significant differences between cases and control as regards TLC, I/T ratio, platelets with high percentage of leukocytosis, leukopenia increased I/T and thrombocytopenia among cases. This finding similar to that of **Du et al. [21]** who noted that the white blood cell count were significant higher in suspected sepsis neonates compared with the corresponding values of controls.

In the present study, there was high percentage of expression of CD64 on neutrophils in patients

when compared with controls and also their percentage of expression was higher in culture positive sepsis than culture negative sepsis. This agrees with **Azza et al. [15]** who reported up regulation of nCD64 in clinical and culture proven sepsis.

In our study, Neutrophil CD64% showed moderate sensitivity (70.7%) and moderate specificity (73.7%) in diagnosis of neonatal sepsis. This is in agreement with **Shi et al. [22]** who found that the pooled sensitivity and specificity of nCD64 were 77 % and 74 %, respectively. And agrees with **Dai et al. [20]** who his results showed that nCD64 is a reliable biomarker for diagnosing neonatal sepsis. The pooled sensitivity and specificity were 80% and 83% respectively.

On the other hand, **Elawady et al. [18]** reported that nCD64 had a sensitivity of 96%, a specificity of 100%, a positive predictive value of 96.2%, and a negative predictive value of 100% in the confirmed and the clinical sepsis groups, respectively.

In the present study, we found that nCD64% cut off level was 17.8. Several studies showed different cutoff values. **Elawady et al. [18]** showed a cutoff value of 45.8 and 46.0 in the confirmed and the clinical sepsis groups, respectively.

On the other hand, **Streimish at al. [16]** showed a cutoff value of 2.19 for late-onset clinical sepsis. While **Kipfmueller et al. [22]** showed a cutoff value of 1.86.

The heterogeneity of sensitivity, specificity and cut-off values of nCD64 in different studies may be explained by the different characteristics of the included patients (preterm or term), definition of sepsis (proven, clinical sepsis), type of sepsis (early, late-onset sepsis) and methods used for measuring nCD64 expression **[23]**.

Study limitation may be relatively small number of different study groups and use of semi quantitative method for measuring CRP.

Conclusion

Results showed that prevalence of infection in neonates is inversely related to gestational age and body weight.

CD64 expression on neutrophils increases significantly in neonates with sepsis and can be considered a useful diagnostic marker for early diagnosis of neonatal sepsis.

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