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Detection of the CD64 on Neutrophils and CD69 on Lymphocytes by Flowcytometry as a Marker for Early Diagnosis of Neonatal Sepsis

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Abstract
Background: *Neonatal sepsis remains one of the main causes of mortality and morbidity despite the progress in hygiene, introduction of new and potent antimicrobial agents for treatment, and advanced measures for diagnosis. The diagnosis of sepsis is difficult because of non-specificity of clinical signs and symptoms and overlapping of symptoms with other noninfectious causes of systemic inflammation.*
Aim: *Identification the CD64 and CD69 markers on the surface of neutrophils and lymphocytes respectively by using flowcytometry assay to evaluate their role in the early diagnoss of bacterial neonatal sepsis.*
Methods: *This study was conducted on 75 neonates who were admitted to Maternity and Childhood Teaching Hospital at neonatal intensive care unit (NICU) at AL-Diwaniyah city, and 75 healthy neonates as a (control group). Flowcytometric analysis was done for detection of CD64 on neutrophils and CD69 on lymphocytes. One ml of EDTA treated blood was freshly processed and analyzed by flowcytometry within 24 hours.*
Results: *The median CD64 neutrophil and CD69 lymphocytes expression was significantly higher among both cases groups compared to healthy controls. In addition, the median for proven sepsis group was significantly higher than that of probable sepsis group.*
Conclusion: *Neutrophil CD64 is a highly sensitive and specific marker for neonatal sepsis. Prospective studies incorporating CD64 into a sepsis scoring system are warranted. However, the important issues of cost and availability are required to be evaluated in routine clinical setting.*
Keywords: *Neonatal sepsis, neutrophil CD64, CD69 lymphocytes, Flowcytometry.*

Introduction

Neonatal sepsis remains one of the main causes of mortality and morbidity despite the progress in hygiene, introduction of new and potent antimicrobial agents for treatment, and advanced measures for diagnosis [(1)]. The diagnosis of sepsis is difficult because of non-specificity of clinical signs and symptoms and overlapping of symptoms

with other noninfectious causes of systemic inflammation [2]. Since 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 [3], several different laboratory determinations are helpful in diagnosis of neonatal sepsis for instances; numerous cell surface antigens have been studied as potentially promising biomarkers of infection, including CD69 and CD64 [4]. Flow cytometric analysis of cell surface antigens (CD11b, CD64, CD32 CD16, CD69, CD25 and CD45) has been performed to detect neonatal sepsis [5]. Other surface markers that have been investigated in different studies include CD69 on peripheral T and B lymphocytes may also have a role [6].

Furthermore, several studies have indicated that quantitation of the neutrophil CD64 is a worthwhile candidate for evaluation as a more sensitive and specific indicator of sepsis than the other available diagnostics tests [7], that has high diagnostic specificity and sensitivity of neonatal sepsis [8,9].

Materials and Methods

Subject: This study was conducted on 75 neonates who were admitted to Maternity and Childhood Teaching Hospital at neonatal intensive care unit (NICU) at AL-Diwaniyah city, and 75 healthy neonates as a (control group) in the period from April to December 2015. They were evaluated for neonatal sepsis with sepsis screen tests, Blood culture, and flowcytometry analysis of CD64 neutrophils and CD69 on lymphocytes. One ml of EDTA treated blood was freshly processed and analyzed by flowcytometry within 24 hours. .By using PE Mouse Anti –Human CD69 and FITC Mouse Anti- Human CD64 (Becton-Dickinson- USA).Neutrophils were electronically selected on the basis of their side – and forward –scatter characteristics and 10.000 cells were analyzed in each sample. Results were expressed as a percentage of positive cells. Considering the percentage of ≥ 20% as positive result while the percentage of < 20% as negative result according to [10]. Informed consent was obtained from all study subjects after explanation of the nature and possible consequences of the study.

Statistical Analysis: Data were translated into a computerized database structure. The database was examined for errors using range and logical data cleaning methods, and inconsistencies were remedied. An expert statistical advice was sought for. Statistical analyses were done using IBMS-PSS version 23 computer software (Statistical Package for Social Sciences) in association with Microsoft Excel 2016. In signal detection theory, a receiver operating characteristic (ROC) is created [11].

Result

The present study were consisting of 2 study groups of new born infants. The patient group were 75 neonates with a clinical diagnosis of neonatal sepsis, which were classified into 2 groups according to the results of blood culture. A positive bacterial culture were 30 cases (Proven sepsis). The culture negative cases were 45(Probable sepsis). And 75 healthy control neonates. No statistically significant differences in gender composition between the 3 study groups. Also, no statistically significant differences between the 3 study groups in proportion of low birth weight and preterm.

The median CD64 neutrophil and CD69 lymphocytes expression was significantly higher among both cases groups compared to healthy controls. In addition, the median for proven sepsis group was significantly higher than that of probable sepsis group, Table(1), Figure (1 and 2).

As shown in Table(2), blood CD64 expression were associated with the highest validity in diagnosing neonatal sepsis and provided a perfect test (area under ROC curve being equal to one). The CD69 lymphocytes expression ranked second in its diagnostic validity and qualified as a good test (ROC area = 0.878).

A positive test (CD64 positive of 30% or higher) is 100% specific and can establish a possible diagnosis of sepsis with 100% confidence in any clinical situation. A negative test on the other hand (obtaining CD64 positive neutrophil expression of <30%) can exclude a possible diagnosis of neonatal sepsis with 99.6% confidence in a clinical situation where neonatal sepsis is of very low possibility (pre-test probability = 10% only), Table (3).

The optimum cut-off value for CD69 lymphocytes expression is set at ≥20%, which is the most accurate cut-off value (91.4%) for this parameter. A positive test at this optimum cut-off value (having a CD69 of 20% or higher) can establish a possible diagnosis of neonatal sepsis with 92.9% confidence in a clinical situation where sepsis is of equal odds (50% pretest probability).

The confidence in a positive diagnosis is further increased to 99.2% when the differential diagnosis of sepsis is highly suspected (90% pre-test probability). A negative test on the other hand (obtaining CD69 positive lymphocytes expression of <20%) can exclude a possible diagnosis of neonatal sepsis with 98.4% confidence in a clinical situation where neonatal sepsis is of very low possibility (pre-test probability = 10% only). Raising the cut-off value further to ≥52% will make the test 100% specific and by extension 100% diagnostic, being able to establish a possible diagnosis of sepsis with 100% confidence in any clinical situation (any pre-test probability), Table(3).

Table (1): The Difference between the Study Groups in Median of CD64 and CD69 Markers.

	Study Group			
	Proven sepsis (Culture positive)	**Probable sepsis (culture negative)**	**Healthy controls**	P
CD64 neutrophil expression				<0.001
Range	(18 to 94)	(3 to 92)	(2 to 19)	
Median	72	60	10	
Inter-quartile range	(60 to 84)	(45 to 68)	(7 to 14)	
No	30	45	75	
Mean Rank	121.3	103.1	40.6	
P (Mann-Whitney) for difference in median between				
Proven sepsis (Culture positive) X Probable sepsis (culture negative) =0.005				
Proven sepsis (Culture positive) X Healthy controls <0.001				
Probable sepsis (culture negative) X Healthy controls <0.001				
CD69 lymphocyte expression				<0.001
Range	(2 to 80)	(3 to 68)	(2 to 50)	
Median	47	39	8	
Inter-quartile range	(28 to 62)	(12 to 45)	(4 to 15)	
No	30	45	75	
Mean Rank	110.9	95.1	49.6	
P (Mann-Whitney) for difference in median between				
Proven sepsis (Culture positive) X Probable sepsis (culture negative) =0.023				
Proven sepsis (Culture positive) X Healthy controls <0.001				
Probable sepsis (culture negative) X Healthy controls <0.001				

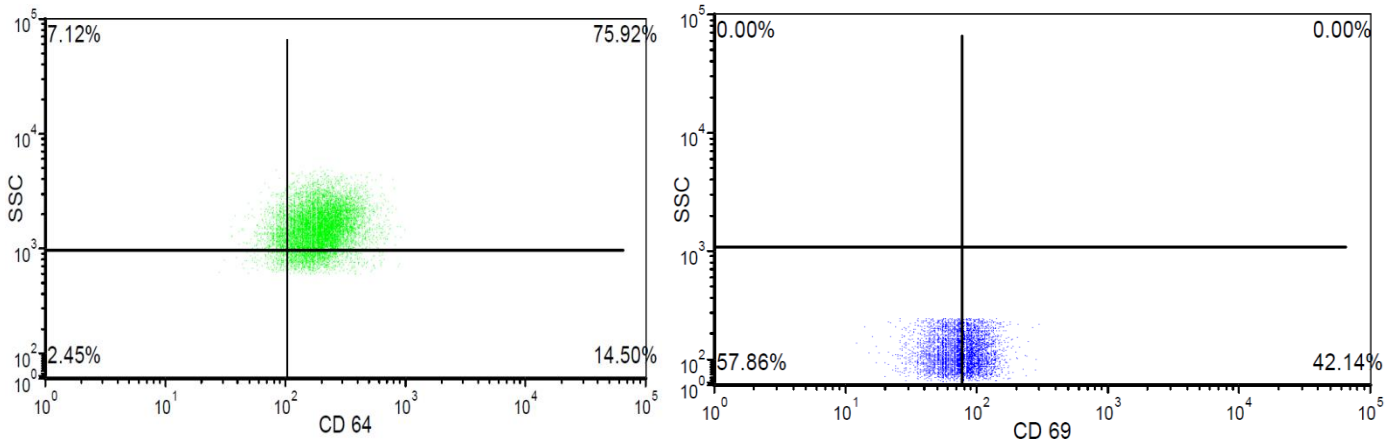

Figure (1): Results of Flowcytometric Analysis for CD64 Detection on Neutrophils (90.42%) Compared with CD69 Detection on T-Lymphocytes (42.14%) in a Patient with Neonatal Culture Proven Sepsis.

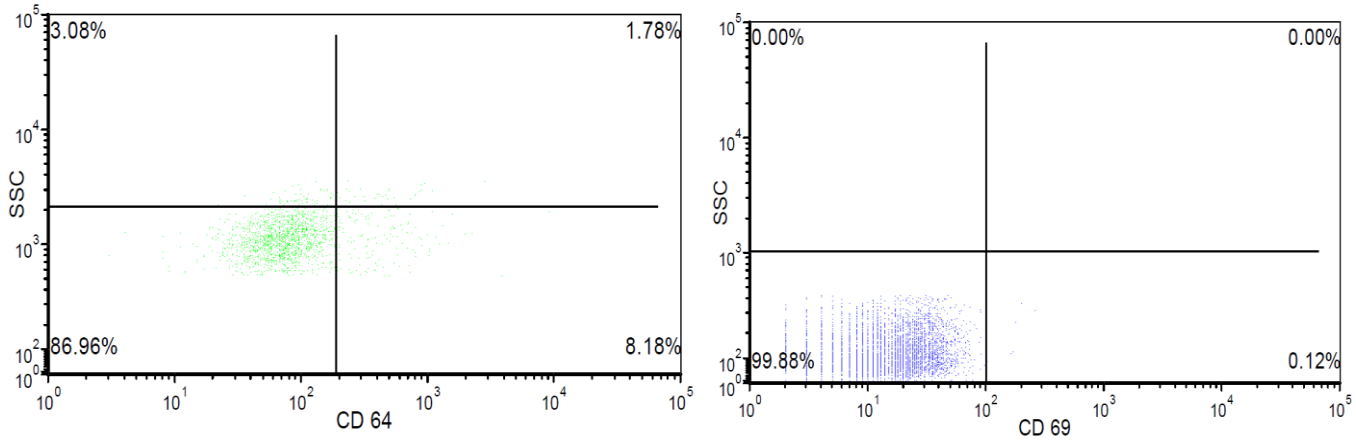

Figure (2): Results of Flowcytometric Analysis for CD64 Detection on Neutrophils (9.96%) Compared with CD69 Detection on T-Lymphocytes (0.12%) in Healthy Neonates.

Table (2): Area Uunder ROC Curve Comparing the Validity of Selected Quantitative Outcome Measurements when Used as Tests to Diagnose Cases with Proven Sepsis (Culture Positive) Differentiating them from Healthy Control Group.

	ROC Area	*P*
CD64 neutrophil expression	0.996	<0.001
CD69 lymphocyte expression	0.878	<0.001

Table (3): Validity Parameters of Selected Quantitative Outcome Measurements when Used as Tests to Diagnose Cases with Proven Sepsis (Culture Positive) are Differentiating them from Healthy Control Group. (Higher Values of the Test are More Suggestive of the Diagnosis).

				PPV at Pre-Test Probability =		*NPV at Pre-Test Probability = 10%*
Positive if ≥ cut-off value	**Sensitivity**	**Specificity**	**Accuracy**	**50%**	**90%**	
CD64 neutrophil expression						
30 (Highest specificity and optimum cut-off)	96.7	100.0	99.0	100.0	100.0	99.6
CD69 lymphocyte expression						
20 (optimum cut-off)	86.7	93.3	91.4	92.9	99.2	98.4
52 (Highest specificity)	43.3	100.0	83.8	100.0	100.0	94.1

Discussion

CD64 is normally expressed in very low concentrations by un stimulated neutrophils, whereas it is considerably upregulated with the onset of bacterial invasion within an hour of acute inflammation [12]. The present work aimed to evaluate the detection of activated surface markers CD64 on neutrophils and CD69 on lymphocytes as diagnostic tool for early identification of a bacterial etiology for neonatal sepsis. High statistically significant difference ($P<0.001$) between patients and controls regarding mean percentage of expression of CD64 on neutrophils, Similar results by Khalifa *et al.*, [13], indicated that quantitation of the neutrophil CD64 is a specific indicator of sepsis than the other available diagnostics tests. Also Similarly Genel *et al.*, [14], founded that there was a highly significant difference between patients group and control group regarding the percentage of CD64 positive neutrophils.

To check diagnosis accuracy, we used the following guidelines based on the AUC level: no informative (0.5), less accurate ($0.5<AUC\leq0.7$), moderately accurate ($0.7<AUC\leq0.9$), and highly accurate ($0.9<AUC<1$), **(**15). Thus, based on our results, CD64 are a highly accurate marker, whereas CD69 is moderately accurate for the diagnosis of neonatal sepsis, Table (2).

In the present study the CD64 neutrophil expression of ≥30% is the optimum cut-off value for this parameter. At this cut-off value the sensitivity was found to be 96.7%, the specificity was 100% (perfect specificity), positive predictive value was 100% and its negative predictive value was 99.6%. A positive test can establish a possible diagnosis of probable sepsis with 100% confidence in any clinical situation. A negative test on the other hand (obtaining CD64 neutrophil expression <30%) can exclude a possible diagnosis of neonatal sepsis with 98.3% confidence in a clinical situation where neonatal sepsis is of very low possibility (pre-test probability = 10% only). Lowering the cut-off value further to ≥3% will result in a perfect sensitivity of 100%. A negative test (CD64 neutrophil expression<3%) can exclude a possible diagnosis of neonatal sepsis with 100% confidence in any clinical situation, Table (3).

In the study of Saiful Islam *et al.*, [16], neutrophil CD64 showed high sensitivity 100%, specificity 54.9%, PPV, 28.13% and also high NPV 100%. Specificity and PPV were low in this study 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. 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. Also This study found that the sensitivity, specificity, positive predictive value and negative predictive value of CD69 lymphocyte expression at 20 (optimum cut-off) were, 86.7, 93.3, 99.2 and 98.4, respectively.

Conclusions

Neutrophil CD64 is a highly sensitive and specific marker for neonatal sepsis. CD69 as T-cell marker activity in the first week of neonatal septicemia. And they may help in diagnosis and follow up of patients.

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