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Early Diagnosis of Neonatal Sepsis: A Molecular Approach and Detection of Diagnostic Markers Versus Conventional Blood Culture

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Abstract: Neonatal sepsis is one of the major causes of neonatal 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 objective of our study was to evaluate the detection of activation surface markers CD64 on neutrophils and CD69 on lymphocytes and using a broad range PCR assay as diagnostic tools for diagnosis of neonatal sepsis of bacterial etiology in comparison with conventional blood culture method. This study was conducted at Microbiology and Immunology Department and Neonatal Intensive Care Unit (NICU), Menoufia University Hospitals. The study population included 45 neonates, 35 patients suspected of sepsis with a mean age of 12.54±7.17 days and 10 healthy controls with a mean age of 13.80±7.46 days. Patients were screened for sepsis by modified clinical sepsis score and hematological sepsis scoring system (total score 7, score ≥ 3 suggestive of sepsis). CBC, semi quantitative CRP, blood culture were done for all neonates. Flow cytometric analysis was done for detection of surface markers CD64 and CD69 on neutrophils and lymphocytes respectively and a broad range PCR assay for detection of bacterial DNA. In the present study, the incidence of culture positive cases (culture proven sepsis) was 26 (74.3%) and culture negative cases (culture unproven sepsis) was 9 (25.7%). Total number of isolates were 26. The most common organism isolated from blood cultures was *Klebsiella* spp. (26.9%). This study showed high expression of CD64 on neutrophils in patients (71.99±18.65) proven sepsis (79.38±7.69) than culture unproven sepsis (50.63±24.55). Neutrophil CD64 showed sensitivity 100%, specificity 66.7%, PPV 89.7% and NPV 100%. The present study showed high statistically significant difference (P =.001) between culture proven sepsis and culture unproven sepsis regarding percentage of expression of CD69 on T-lymphocytes. On evaluation of PCR status in relation to blood culture in studied cases, PCR revealed a 73.1% sensitivity, 100% specificity, 100% positive predictive value and 56.3% negative predictive value. In the present study, CRP results did not differentiate neonatal sepsis patients with bacterial infections from those with no bacterial infections. In conclusion, flow cytometric assessment of neutrophil CD64 may find its role in ruling out bacterial sepsis. Low sensitivity and NPV of broad range PCR were detected in this study reflecting that for this method to replace or supplement blood culture in early diagnosis of bacterial sepsis, it needs to be further developed and improved.

Key words: CD64 · Neonatal sepsis · Blood culture · Broad range PCR

INTRODUCTION

Neonatal sepsis is an invasive infection occurring within the first 28 days of life. It occurs in a range of 1-10/1000 live births and despite advances in perinatal care, neonatal sepsis is still a significant cause of morbidity and mortality [1]. Diagnosis of neonatal sepsis is difficult to establish and remains a challenge for neonatal health care providers [2]. The gold standard for detecting bacterial sepsis is blood culture [2]. However,

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as pathogens in blood cultures are only detected in approximately 25% of patients, the sensitivity of blood culture is suspected to be low [3]. This leads to unnecessary exposure to antibiotics before the presence of sepsis has been proven with potentially poor outcomes. Clinicians have long sought reliable markers to detect sepsis early in its course and to exclude diseases of noninfectious origin [3]. Recently, flow cytometric analysis of cell surface antigens (CD11b, CD64, CD32, CD16, CD69, CD25 and CD45) has been performed to detect neonatal sepsis [4]. CD64 is glycoprotein, known as FC gamma receptor 1 (FC RI), plays a coordinating role in immunity and mediates functions such as endocytosis, phagocytosis and cytokine production [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]. In recent years, PCR analysis has exploited the highly conserved bacterial 16S ribosomal ribonucleic acid (rRNA) gene to diagnose neonatal sepsis [7]. The broad range PCR analysis relies on the fact that the bacteria specific 16S rRNA gene is highly conserved in all bacterial genomes, is a useful method for identification of bacteria in clinical samples. Amplification targeting of this 16S rRNA gene is a potentially valuable clinical tool in samples with low copy numbers of bacterial DNA, as this gene is present at 1 to more than 10 copies in all bacterial genomes [8]. The present work aimed to evaluate the detection of activation of surface markers CD64 on neutrophils and CD69 on lymphocytes as well as using a broad range PCR assay for detection of bacterial DNA as a new diagnostic tool for early identification of a bacterial etiology for neonatal sepsis and comparing both recent investigated methods with conventional blood culture method.

MATERIALS AND METHODS

Subjects: This study was conducted at the Microbiology and Immunology Department, in collaboration with Neonatal Intensive Care Unit (NICU), Menoufia University Hospitals.

Subjects were classified into two groups :

Group 1: Included 35 patients (14 females and 21 males) aged from 1-28 days. They were screened for sepsis by modified clinical sepsis score [9] and hematological sepsis scoring system and interpreted as suspected cases of sepsis (total score 7, score ≥ 3 is suggestive of sepsis) [10].

Group 2: Included 10 apparently healthy controls neonates (4 females and 6 males), matched by age and sex with the patients.

Methods: After their parents consent, patients were subjected to full medical history taking including prenatal, natal and postnatal history and invasive procedures that were done to the baby after delivery. Full clinical examination for early and late symptoms and signs of sepsis. Laboratory investigations included complete blood picture (CBC) and semi quantitative C- reactive protein (CRP).

Bacteriological Examination: Blood culture using blood culture bottles (Egyptian Diagnostic Media) was performed for all patients then incubated at 37°C for up to 7 days, under aerobic condition. Subculture was done on 3rd, 5th and 7th day on blood agar, chocolate agar, mannitol salt agar and MacConkey's agar plates. Colonies grown on different media were subjected to further morphological and biochemical identification according to the standard microbiological methods [11].

Flow Cytometry: Flow cytometric analysis was done for detection of CD64 neutrophils and CD69 on lymphocytes. One ml of EDTA treated blood was freshly processed and analyzed by flow cytometry within 24 hours. To block Fc receptors, 34 ul of a 3 mg/ml solution of normal mouse IgG (Caltag/Burlingame, CA) was added to each tube. Samples were then liquated into required tubes of 100 ul each and stained with 5 ul of appropriate combination of monoclonal antibodies to membrane markers (CD3 FITC, CD69 PE) (CD22 FITC, CD69 PE) and CD64 FITC *(CytoMol, USA*). Cells were incubated for 15 minutes on ice. Erythrocytes were lysed using three ml of pre-warmed lysing reagent and centrifuged three minutes at room temperature. After discarding the supernatant and re-suspending the cells in residual buffer the cells were fixed in 200 ul of 2% ultra-pure formaldehyde. Data was acquired using a FACS Caliber flow cytometer (BD immune cytometry systems, San Jose, CA). Data was analyzed using Win list (verity Software House, Topsham. ME). The gating strategies were as the following; in the forward scatter (FSC) and side scatter (SSC) histogram, we made three regions, R1 {lymphocytes (red color)} low FSC and low SSC, R3 {Neutrophilis (blue color)} high FSC and high SSC and R2 { monocytes (green color) } in between. R1 was further analyzed in quadrant histogram for T lymphocytes (CD3 FITC, CD69 PE) and B lymphocytes

(CD22 FITC, CD69 PE) and R3 was further analyzed in quadrant histogram for neutrophils expressed CD64 FITC. For each sample, 5000 events were recorded. Results were expressed as percentages.

DNA Amplification Technique: A broad range PCR assay for detection of bacterial DNA in blood was performed. Bacterial DNA was extracted from whole blood by using QIA amp DNA Mini Kit (Qiagen, Germany) [12]. Amplification was carried out in a thermal cycler (Biometra, Germany). Two oligonucleotide primers were used,5'-TGAAGAGTTTGATCATGGCTCAG-3'(forward primer) and (reverse primer) 5'- TACCGCGGCTGCTGGCA 3', (Qiagen, Germany). The primers react with highly conserved regions of the bacterial 16S rRNA gene to provide PCR products of approximately 500 base pairs. Each PCR reaction (50 µl) consisted of 1 × Amplitaq Gold buffer supplemented with 2.5 U Taq polymerase enzyme, 0.5 uM each primer, 2 mM MgCl2, 0.2 mM dNTP, 20 µl template and PCR distilled water. Cycling conditions included a 5 minute initial denaturation step at 94°C followed by 35 cycles of 20 seconds at 94°C, 20 seconds at 58°C and 60 seconds at 72°C. This was followed by one cycle of final extension for 10 minutes at 72°C [13]. The amplified PCR products were detected by agarose-gel electrophoresis [13]. Regarding the controls, only blood samples were taken and subjected to the following CBC, CRP detection, flow cytometric analysis for detection of CD64 and CD69 on neutrophils and lymphocytes respectively.

Statistical Analysis: Mann-Whitney test was used for comparison between two groups having quantitative variables. P-value of < 0.05 is statistically significant.

- Sensitivity= a/ (a+c) - Specificity= d/ (b+d)
- Negative predictive value= d/ (c+d)
- Positive predictive value= a/ (a+b)
- a= true positive cases
- b= false positive cases
- c= false negative cases
- d= true negative cases

RESULTS

Our study included 45 neonates, 35patients with a mean age of 12.54±7.17 days and 10 controls with a mean age of 13.80±7.46 days. In patient group, 21 were males (60%) and 14 were females (40%), while in controls, 6 neonates were males (60%) and 4 were females (40%) with no significant differences (P>0.05) between the patients and controls regarding both age and sex. In patient group, male cases were more than females, premature and low birth weight cases (24 cases, 68.6% each) were more than full term and normal birth weight (> 2500 g) (Table 1).

Table 1: Demographic criteria and risk factors for sepsis of studied cases.

	Case (n = 35)	
Parameters	NO	%
Sex		
Male	21	60
Female	14	40
Birth weight / g		
Birth weight >2500 g	11	31.4
Low Birth weight (1000 - 2500 g)	24	68.6
Prematurity		
Preterm	24	68.6
Full term	11	31.4
Mode of delivery		
Normal delivery	16	45.7
Cesarean section	19	54.3
Exposure to invasive procedures		
Positive	18	51.4
Negative	17	48.6
Maternal risk factors		
Premature rupture membrane (> 18 hours)		
Positive	18	51.4
Negative	17	48.6
Intra partum Maternal fever >38°C		
Positive	4	11.4
Negative	31	88.6
Maternal Infection		
Clinical chorioamnionitis	5	14.3
¤Other infections	5	14.3
Negative	25	71.4

Culture positive cases (culture proven sepsis) were 26 (74.3%). Total number of isolates were 26, Gram negative bacteria were more frequent than Gram positive bacteria with a frequency of 15 (57.7%) and 11 (42.3%) respectively. The most prevalent bacteria was *Klebsiella* spp. (26.9%). In our study, there were no significant differences between culture proven sepsis and culture unproven sepsis cases regarding gestational age, prematurity, birth weight, mode of delivery and exposure to invasive procedures (Table 2).

There were significant differences (P<0.5) between culture proven sepsis and culture unproven sepsis cases regarding white blood cells count and hemoglobin level. While the differences in total neutrophils%, immature neutrophils%, the ratio of immature to total neutrophils and platelets count between the two groups were not statistically significant (P>0.05) (Table 3).

Table 2: Comparison between cases of culture proven and culture unproven sepsis in relation to demographic criteria and risk factors

	Blood culture					
	Positive (n = 26)		Negative (n = 9)		Test of significance	P value
GA / weeks X±SD	33.88±3.96		35.55±3.97		Mann Whitney U test 1.0	0.31 NS
Birth weight / g X±SD	2068.35±711.08		2241.11±347.96		Mann Whitney U test 0.64	0.52 NS
	No	%	No	%		
Prematurity						
Preterm	18	69.2	6	66.7		
Full term	8	30.8	3	33.3	Fisher's Exact test 0.02	1.0 NS
Mode of delivery						
NVD	11	42.3	5	55.6		
CS	15	57.7	4	44.4	Fisher's Exact test 0.47	0.70 NS
Exposure to invasive procedures						
Positive	13	50.0	5	55.6		
Negative	13	50.0	4	44.4	Fisher's Exact test 0.08	1.0 NS
Maternal risk factors						
PROM (> 18 hours)						
Positive	13	50.0	5	55.6		
Negative	13	50.0	4	44.4	Fisher's Exact test 0.08	1.0 NS
Intra partum Maternal fever >38°C						
Positive	3	11.5	1	11.1		
Negative	23	88.5	8	88.9	Fisher's Exact test 0.001	1.0 NS
Maternal infection						
Clinical chorioamnionitis	3	11.5	2	22.2		
¤Others	4	15.4	1	11.1		
Negative	19	73.1	6	66.7	X^2 0.66	0.72 NS

Table 3: CBC results of studied culture proven and culture unproven cases of sepsis

	Blood culture			
	Positive (n = 26)	Negative (n = 9)	Mann Whitney U test	P value
Hb gm/dl X±SD	13.72±1.83	12.53±2.10	1.49	0.014 S
WBC (10^3/mm^3) X±SD	16.98±7.58	8.46±6.01	2.90	0.004 HS
Total PMN% X±SD	49.42±7.50	47.44±5.72	0.51	0.61 NS
Immature PMN% (bands or staff) X±SD	11.5±6.23	8.44±4.92	1.17	0.24 NS
I : T ratio X±SD	0.23±0.13	0.17±0.08	1.19	0.23 NS
Platelets (10^3/mm^3) X±SD	194.73±84.75	161.66±87.35	0.91	0.36 NS

P = probability of error X = mean SD = standard deviation
HS = highly significant NS = non significant Hb = hemoglobin level
I : T ratio = ratio of immature to total neutrophils

Table 4: CRP results of studied cases and controls

	The studied groups					
	Case (n = 35)		Control (n = 10)		Test of significance	P value
CRP (mg/L) X±SD	32.91±25.42		7.50±2.12		2.50	0.012 S
	No	%	No	%		
CRP						
Positive	35	100	2	20.0		
Negative	0	0.0	8	80.0	34.05	<0.001 HS

On analysis of CRP results, its levels were significantly higher in patients (32.91±25.42 mg/L) than controls (7.5±2.12 mg/L) (Table 4).

Flow cytometric analysis was done for detection of surface markers CD64 and CD69 on neutrophils and lymphocytes respectively. In the present study, there was high percentage of expression of CD64 on neutrophils in patients (71.99±18.65%) when compared with controls (5.37±3.54%) and also their percentage of expression was higher in culture proven sepsis (79.38±7.69%) than culture unproven sepsis (50.63±24.55%) (Table 5, 6 and Figure 1 and 2). The present study revealed high significant difference between patients (2.11±.1.07%) and controls (0.83±0.67%) regarding mean percentage of expression of CD69 on T-lymphocytes and B-lymphocytes (Table 5), but only the expression of CD69 on T-lymphocytes could

Table 5: Results of activation of surface markers detected by flow cytometer in studied cases and controls

	The studied groups			
Activation surface marker	Case (n = 35)	Control (n = 10)	Mann Whitney U test	P value
CD64 on Neutrophils% X±SD	71.99±18.65	5.37±3.54	4.77	0.001 HS
CD69/3 On T-Lymphocytes% X±SD	2.11±.1.07	0.83±0.67	3.42	0.001 HS
CD69/22 On B-Lymphocytes% X±SD	1.14±0.60	0.68±0.33	2.28	0.022 S

Table 6: Results of activation surface markers detected by flow cytometer in culture proven and culture unproven cases of sepsis

	Blood culture			
Activation surface marker	Positive (n = 26)	Negative (n = 9)	Mann Whitney U test	P value
CD64 on Neutrophils% X±SD	79.38±7.69	50.63±24.55	3.19	0.001 HS
CD69/3 On T-Lymphocytes% X±SD	2.34±1.09	1.48±0.77	2.26	0.024 S
CD69/22 On B-Lymphocytes% X±SD	1.10±0.51	1.21±0.85	0.05	0.95 NS

Table 7: Performance of diagnostic tests or combination of tests for neonatal bacterial sepsis

Parameter	Sensitivity	Specificity	Positive predictive value	Negative predictive value
Activation Surface Marker CD64	100%	66.7%	89.7%	100%
CD69/3	80.8%	66.7%	87.5%	75.0%
CD69/22	84.6%	44.4%	84.6%	55.6%
PCR	73.1%	100%	100%	56.3%
CRP	100%	0.0%	74.3%	0.0%
Combined Tests				
CD64 + CD69/3	100%	44.4%	83.9%	100%
CD64 + CD69/22	100%	22.2%	78.8	100%
CD69/3 +CD69/22	94.3%	50%	86.8%	71.4%

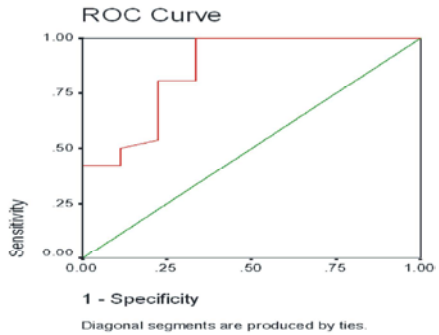

Fig. 1: Receiver operating characteristic (ROC) curve of CD64 as a diagnostic test using blood culture as a gold standard. This figure shows: Area under the curve (AUC) = 0.86 (0.001) at cutoff point 62.36 Sensitivity = 100%, Specificity = 66.7%, Positive predictive value = 89.7%, Negative predictive value = 100%

differentiate culture proven and culture unproven sepsis groups and not its expression on B-lymphocytes (Table 6 and Figure 3, 4).

Using the blood culture for diagnosis of infected neonates as the gold standard, neutrophil CD64 showed sensitivity 100%, specificity 66.7%, PPV 89.7% and NPV 100% (Figure 1). On the other hand, CD69/3 and CD69/22 had high sensitivity (80.8% and 84.6% respectively) but low NPV. Combination of the studied markers such as CD64 + CD69/3, CD64 + CD69/22 and CD69/3 + CD69/22 was associated with both higher sensitivity and better negative predictive values (Table 7). On evaluation of PCR status in relation to blood culture, 19 of 26 blood culture positive cases were also positive by PCR, while 7 of 26 blood culture positive cases were negative by PCR. PCR revealed a 73.1% sensitivity, 100% specificity, 100% positive predictive value and 56.3% negative predictive value (Table 7). There were seven patients with blood culture positive PCR negative, their blood cultures showed *Staph. aureus* (1 case), *E. coli* (2 cases) and *Klebsiella* spp. (4cases). Clinical and haematological sepsis scores of these seven patients were in favor of diagnosing them as neonatal sepsis.

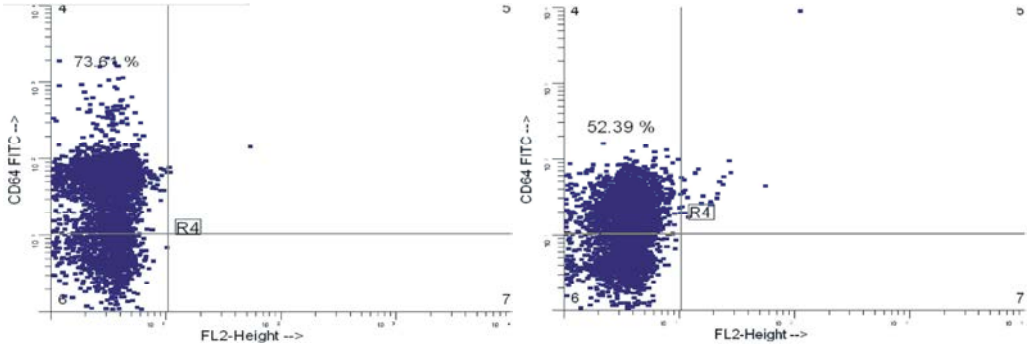

Fig. 2: Results of flow cytometric analysis for CD64 detection on neutrophils in a patient with neonatal culture proven sepsis (73.61%) compared with culture unproven sepsis (52. 39%)

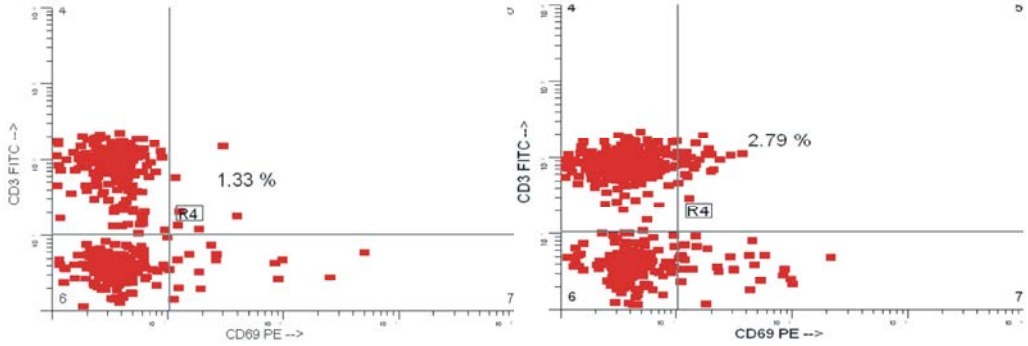

Fig. 3: Results of flow cytometric analysis for CD69 detection on T-lymphocytes in a patient with neonatal culture proven sepsis (2.79%) compared with culture unproven sepsis (1.33%).

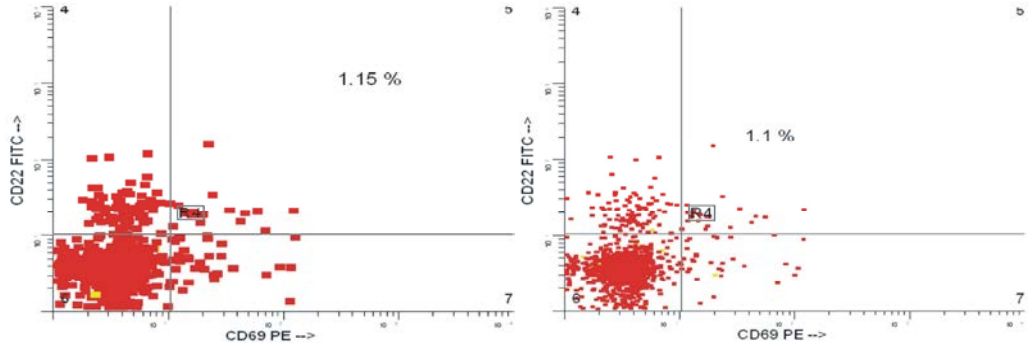

Fig. 4: Results of flow cytometric analysis for CD69 detection on B-lymphocytes in a patient with neonatal culture proven sepsis (1.15%) compared with culture unproven sepsis (1.1%).

DISCUSSION

Several novel tests show promise in the early detection of neonatal sepsis. In the present study we tried to highlight new combinations of cell surface markers and identification of sepsis based on gene expression profiling. Although blood culture is the gold standard for the diagnosis of sepsis, culture reports would be available only after 48-72 hours [14]. This study included 35 patients with a mean age of 12.54±7.17 days. Premature and low birth weight (68.6% each) were more than full term and normal birth weight (> 2500 g) cases. This results are in agreement with Jiang *et al*. [15], that the majority of sepsis episodes occurred in LBW (75%) and premature infants (76.7%). Immature host defense mechanisms and invasive life support systems make the premature neonate particularly susceptible to overwhelming infection [16, 17]. In the present study, culture proven sepsis cases were

26 (74.3%) and culture unproven sepsis cases were 9 (25.7%). Some studies showed higher blood culture positivity as Mondal *et al*. [18], (blood culture was positive in 61.3% of cases). Other studies showed lower culture positivity as Shah *et al*. [19], (31.57%) and Hussein *and* Khaled [20], (42%). In our study, the mean CRP level was significantly higher in patients (32.91±25.42) than controls (7.5±2.12). In agreement to our results, Mondal *et al*. [18], founded that the mean CRP level was significantly higher in patients than controls. CRP test had 100% sensitivity, 0.0% specificity, 74.3% PPV and 0.0% NPV. CRP has been evaluated by many workers but the lack of specificity was the main disadvantage. In accordance with our results Chan [21], reported that CRP measurement in neonatal sepsis had sensitivity and specificity, positive and negative predictive values as 56%, 72%, 71% and 57% respectively. The quantitative CRP values, particularly when repeated, are highly specific and have good sensitivity [21]. In addition, serial measurements can be helpful in monitoring the response to treatment. CRP still remains the preferred index in most NICUs [22]. 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. Flow cytometric analysis has the advantage over conventional immunological assay methods being able to localize the activated markers to a specific cell type. Our study showed high statistically significant difference (P=.001) between patients and controls regarding mean percentage of expression of CD64 on neutrophils. Similar results by Khalifa *et al*. [24], indicated that quantitation of the neutrophil CD64 is a specific indicator of sepsis than the other available diagnostics tests. The present study showed high statistically significant difference (P=.001) between culture proven sepsis and culture unproven sepsis regarding percentage of expression of CD64 on neutrophils and significant difference between both groups regarding percentage of expression of CD69 on T-lymphocytes. Similarly Genel *et al*. [25], 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 infection/sepsis, as the quantitation of neutrophil CD64 is rapid (<60minutes) and only minimal blood volume is used, which is a real advantage in neonates [26]. In the present work, neutrophil CD64 showed better sensitivity, specificity, PPV and NPV (100%, 66.7%, 89.7% and 100% respectively). The high negative predictive value of neutrophil CD64 indicates that it may find its role in ruling out sepsis/infection. Our findings coincided with the outcome of numerous studies done on the diagnostic performance of neutrophil CD64 in sepsis in view of the high sensitivity compared to blood culture and high negative and positive predictive values [27]. In our study, combination of the studied markers such as CD64 + CD69/3, CD64 + CD69/22 and CD69/3 + CD69/22 was associated with both higher sensitivity and better negative predictive values. Recent investigations have focused on the combination of markers ensuring greater diagnostic accuracy. In a previous work by *Ng et al*. [28], the use of CD64 in combination with other diagnostic markers such as CRP improved the sensitivity and negative predictive value. There has been great interest in the development of PCR tests, which detect the conserved 16S region of the bacterial genome [28]. Early exclusion of bacterial infection could help to reduce overuse of antibiotics [29]. Our study showed that 19 of 26 blood culture positive cases were also positive by PCR, while 7 of 26 blood culture positive cases were negative by PCR. Compared to blood culture the diagnosis of bacterial proven sepsis by PCR revealed a 73.1% sensitivity, 100% specificity, 100% positive predictive value and 56.3% negative predictive value. Comparable results obtained by *Reier-Nilsen et al*. [7] reported sensitivity, specificity, PPV and NPV of 66.7%, 87.5%, 95.4% and 75%, respectively, using 16S rRNA targeted PCR. In disagreement to our study, Yadav *et al*. [30], compared broad-range PCR with blood cultures in 100 neonates with suspected sepsis, PCR assay had 100% sensitivity and 95.6% specificity. The possible reason for frequently reported so-called 'false' positives in which PCR shows evidence of pathogen DNA in the absence of culturable organisms is low copy number of bacteria in cultured sample [17].

In conclusion, flow cytometric assessment of neutrophil CD64 can be considered a valuable marker for diagnosis of bacterial neonatal sepsis. CRP results did not differentiate neonatal sepsis patients with bacterial infection from those with no bacterial infection. Low sensitivity and NPV of broad range PCR were detected in this study reflecting that for this method to replace or supplement blood culture in early diagnosis of bacterial sepsis, it needs to be further developed and improved.

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