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RESEARCH ARTICLE

EVALUATION OF CD64 DETECTION ON NEUTROPHILS AND TLR-2 ON MONOCYTES BY FLOWCYTOMETRY AS MARKERS FOR EARLY DIAGNOSIS OF NEONATAL SEPSIS

Fatma Mostafa Mahmoud[1],Nebal Medhat Darwish[1], Rania Ahmed Hassan[1], Nancy Mohamed Abou Shady[2]
1.Department of Medical Microbiology and Immunology.
2.Department of Pediatrics Faculty of Medicine – Ain Shams University

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Corresponding Author

Rania Ahmed Hassan

Abstract

Neonatal sepsis remains one of the main causes of neonatal mortality.Early diagnosis and treatment of neonatal sepsis may help to decrease neonatal mortality. However,blood culture results are not available for 48 hours. A reliable marker or set of markers is required for prompt identification of neonatal sepsis.CD64 expression on neutrophilsisup regulated within six hours after their activation. Quantitation of the neutrophil CD64 is a candidate for evaluation as a sensitive and specific indicator. Another proposed marker is TLR-2 whose expression on monocytes/phagocytes increases at the initial presentation of sepsis.

Aim of the study: To evaluate detection of CD64 on neutrophil and TLR-2 on monocytes by flowcytometry as early markers for diagnosis of neonatal sepsis.

Methods: This study included 50 neonates selected from the NICU, Ain Shams University pediatric Hospital: 25 neonates suspected clinically as neonatal sepsis patients, and 25apparently healthy neonates served as a control group. Blood cultures were done to confirm the diagnosis in the patient's group. Detection of CD64 and TLR-2 on surfaces of neutrophils and monocytes respectively was done by flowcytometry in blood samples of both groups.

Results: A significant increase in CD64 expression was found in patients than in control groups.The combination of CRP and TLR-2 showed sensitivity (100%), specificity (50%), positive predictive values (75%), and negative predictive values (100%).

Conclusion: A combination of two markers has better performance in the diagnosis of neonatal sepsis. However blood culture remains irreplaceable to confirm the diagnosis and perform antimicrobial susceptibility.

Introduction

Neonatal sepsis is defined according to the 2002 International Pediatric Sepsis Consensus Conference, as systemic inflammatory response syndrome in the presence of or as a result of suspected or proven infection, But so

far, a worldwide agreement on the definition of early-onset neonatal sepsis (EONS) has not been reached **(Ying and Jia-Lin 2012) .**

Infection in the neonate may be categorized as early onset sepsis (EOS) (<72hours of age) or late onset sepsis (LOS) (>72hours of age) **(Groselj _Grenc et al., 2009).**

Early onset sepsis is caused by organisms prevalent in the maternal genital tract, infecting the neonate transplacentally or during passage through a colonized birth canal at delivery **(Anderson-Berry et al., 2010).** The micro-organisms most commonly associated with EOS include group B Streptococcus (GBS), Escherichia coli (E. coli), Haemophilusinfluenzae and Listeria monocytogenes**(Tripathiand Malik, 2010).**

The LOS is acquired from the caring environment (hospital). Micro-organisms associated with LOS include coagulase-negative Staphylococci, Staphylococcus aureus, E coli, Klebsiella, Pseudomonas, Enterobacter, Candida, GBS, Serratia, Acinetobacter, and anaerobes **(Anderson-Berry et al., 2010).**

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 **(Naher and Khamael, 2013)**. So it is responsible for 30- 50% of the total neonatal deaths in developing countries. It is estimated that up to 20% of the neonates develop sepsis and approximately 1% die of sepsis related causes **(Gandhi et al., 2013).**

The prognosis and outcome of neonatal sepsis depend on early diagnosis and efficient antibiotic therapy **(Stoll,2007),** so early diagnosis and treatment of neonatal sepsis may help to decrease neonatal mortality. However early identification of neonatal sepsis is difficult because of the nonspecific or minimal clinical presentations. The clinical course of neonatal sepsis can be fulminant within hours of onset **(Ng et al., 1997)**, and infection in the newborn period is associated with 10% of neonatal death **(Stoll et al., 1998).** Thus, it is extremely important to make an early and accurate identification of neonatal sepsis for prompt antimicrobial therapy and better outcomes.

The gold standard for confirming diagnosis of neonatal sepsis is blood culture. However, blood culture results are not available for 48 hours after starting the culture, and if blood cultures are drawn after administration of antibiotics, growth of microorganisms can be suppressed **(Icardi et al., 2009).** Hence, a reliable inflammatory marker or set of markers is required for prompt and accurate identification of neonatal sepsis, so that delayed or unnecessary treatment can be avoided **(Young et al.,2012).**

Numerous cell surface antigens have been studied as potentially promising biomarkers of infection, including CD11b, CD69 and CD64 **(Ng and Lam, 2006).** CD64 is a 72-kDa glycoprotein, known as FC gamma receptor-1 (FC_γ RI) that binds immunoglobulin G (IgG) with high affinity **(Masuda and Roos, 1993).**

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 neutrophils **(Groselj _Genc et al., 2009).** During neutrophil activation, under the influence of inflammatory cytokines (Interleukin-12, Interferon gamma-INF_γ and Granulocyte colony stimulating factor-G-CSF), there is up regulation of neutrophil CD64 (Davis et al., 2006). Up regulation of CD64 occurs within four to six hours after stimulation with $interferon_\gamma$ or granulocyte colony stimulating factor **(Allen et al., 2002).**

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 **(Davis et al., 2006).** The majority of the studies agree that neutrophil CD64 has high diagnostic specificity and sensitivity of neonatal sepsis **(Cardelli et al., 2008, Bhandari et al., 2008).**

Toll-like receptors (TLRs) are a family of pattern recognition receptors (PRRs), which act as key components of the innate immune response by recognizing pathogen associated molecular patterns (PAMPs) **(Rameriz et al.,2012).**

Toll-like receptors (TLRs) play a crucial role in the initiation of adaptive responses to bacteria invasion. A critical feature of macrophages required for specific recognition and response includes the expression of optimum levels of different TLRs. Upon the interaction with specific pathogen-associated molecular patterns (PAMPs), TLRs trigger the signal transduction cascades that lead to cellular responses including the engulfment of invasive microbes, induction of inflammatory cytokines, and generation of reactive oxygen species (ROS) to kill the invasive pathogens **(Westet al., 2011).**

A low basal expression of TLR-2 might be associated with the particular susceptibility of neonates for infections with Gram-positive pathogens. After infection, the expression of TLR-2 on monocytes/phagocytes increases at the initial presentation of clinical symptoms, and shows a constant high expression in the course of neonatal sepsis, and is down regulated after successful treatment **(Umlauf et al., 2013).**

The expression levels of the Toll-like receptor (TLR)2 on monocytes by flow cytometry, is comparably more valuable than C reactive protein, IL-8, and IL-6 as early neonatal sepsis markers **(Bhat and Rao., 2010).**

SUBJECTS AND METHODS

This study was conducted on 50 neonates selected from the Neonatal Intensive Care Unit (NICU), Ain Shams University pediatric Hospital, during the period from December 2012 to February 2013. They were divided into two groups:Group 1 (patients' group): It included 25 neonates (11 females and 14 males) aged between 1-28 days with mean age13.32±7.02, selected according to the following criteria:

Gestational age > 34 weeks and birth weight > 1200 g.

Signs of sepsis: fever, tachycardia, tachypnea, grunting, signs of respiratory compromise, signs of cardiovascular compromise and signs of metabolic changes **(Young et al., 2012).**The severity of sepsis was assessed according to Radwell score**(Narasimba and Harendra, 2011).**

Criteria	Abnormality	Score
Total WBC count	≤ 5,000/dl	1
	≤30,000—12–24 h ≤25,000 at birth ≤21,000—Day 2 onwards	1
Total PMN count	No mature PMN seen	2
	Increased/decreased	1
Immature PMN count	Increased	1
I:T PMN ratio	Increased	1
Degenerative changes inPMN	Toxic granules/cytoplasmic vacuoles	1
Platelet count	≤ 150,000/dl	1

The normal values are:
Total PMN count —1800–5400
Immature PMN count—600
Immature: Total PMN ratio—0.120
Immature: Mature PMN ratio—<0.3

Patients who were < 34 weeks, <1200g were excluded from the study.

Group 2(control group): It included 25 apparently healthy neonates (9 females and 16 males), matched by age with patients group.

All neonates were subjected to full history taking, thorough clinical examination, Laboratory investigations which include Complete blood count (CBC) using blood samples collected in EDTA tubes by coulter counter T660 with differential leucocytic count using Leishman-stained blood smear, c-reactive protein (CRP) estimated by latex agglutination assay.

A 2 ml blood sample was withdrawnunder complete aseptic conditions, and was divided as follows: 1 ml of blood was inoculated to an 8 ml broth containing blood culture bottle supplied by Egyptian Diagnostic Media (EDM). And 1 ml was added to an EDTA -containing sterile tube for flow cytometric analysis to detect the activation surface markers (CD64,TLR-2). The sample was freshly processed and analyzed within 24 hours.

The inoculated blood culture bottles were incubated under aerobic conditions and subcultured on 3rd,5th and 7th days. Colonies were subjected to further morphological and biochemical identification to identify different bacterial species according to the standard microbiological methods **(Koneman et al., 2006).**

Detection of CD64 and TLR2 on neutrophils and monocytes respectively:

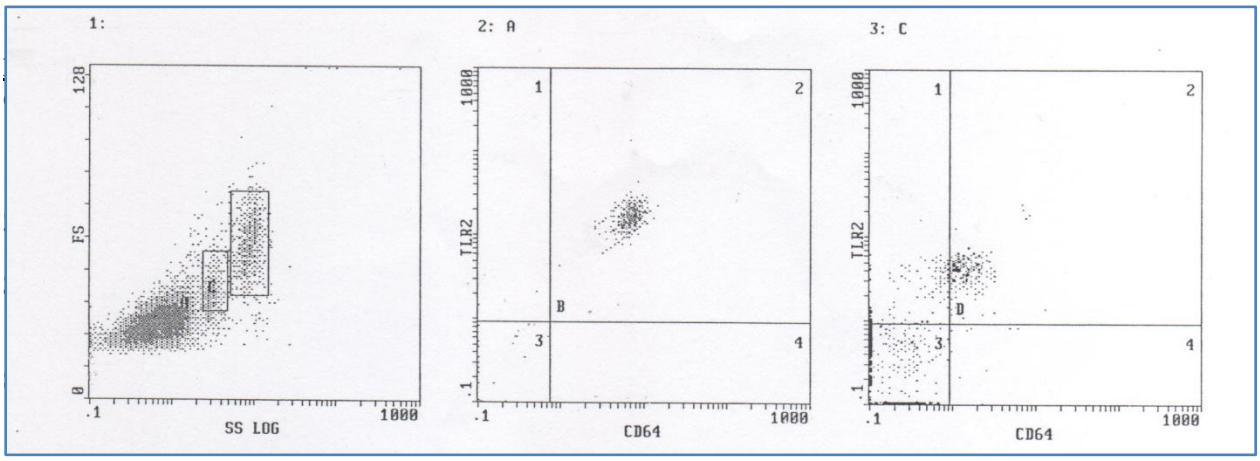
This was done usingflow cytometry device (Coulter Epics XL TM USA, 1999) flow cytometer (Miami, Florida, System II, TM software), andIOTest CD64-FITC Conjugated Antibody - 2ml Liquid - 20 microliter per test, clone 22 (Beckman Coulter ,USA), and human TLR2 PE conjugated antibodies - 2ml liquid -20 microliter per test ,clone # 383936 (R&D systems ,USA).

The sample was washed three times in an isotonic phosphate buffer (supplemented with 0.5% BSA) followed by centrifugation at 500xg for 5 minutes to remove contaminating serum components.5µL of each type of monoclonal antibodies were added to 50µL of whole blood and incubated at 4°c for 15 minutes in dark.1.5ml of lysis solution were added, the tube was vortexed well for complete hemolysis.Then the cells were washed twice in 4mL of the same PBS buffer to remove unreacted anti-CD64 and TLR2 reagent.Cells were then re-suspended in 200 - 400 µL of PBS buffer for final flow cytometric analysis.

Cell surface expression of CD64 was determined at 468 nm wavelength laser excitation and the emitted fluorescence was monitored with a detector optimized to collect peak emissions at 504 - 541nm, while cell surface expression of TLR-2 was determined at 488 nm wave length laser excitation and the emitted fluorescence was monitored with a detector optimized to collect peak emissions at 565-605nm.

Neutrophils and monocytes phenotyping was done by gating according to forward scatter (size) and side scatter (granularity) strategy.

Results were expressed as percentages of cells positive for CD64 and TLR-2.



Analysis of data was done by IBM computer using SPSS (statistical program for social science version 12).Quantitative variables are described as mean, SD and range. Qualitative variables expressed as number and percentage. Chi-square test was used to compare qualitative variables between groups. Unpaired t-test used to compare quantitative variables, in parametric data. Mann Whitney test was used in non-parametric data. p value <0.05 was considered significant.Spearman Correlation co-efficient test used to rank variables versus each other positively or inversely. ROC (receiver operator characteristic curve) was used to find out the best cut off to test the validity of certain variables (sensitivity,specificity, positive and negative predictive values).

RESULTS

The present study included 25 newborns with sepsis; 14males (56%) and 11 females (44%), their mean age was 13.32 ± 7.02 and 25 healthy controls; 16 males (64%) and 9 females (36%), with a mean age of7.08 ± 1.82. Both groups were statistically matched.

The laboratory investigations in this study showed that the patients had high WBCs count, high ANC, high I \T, mild decrease in platelets count, slight low hemoglobin level, and high C- reactive protein. 15/25 (60%) of septic cases were blood culture positive, while 10/25 (40%) of septic cases were blood culture negative (Table 1).

In this study, the total number of isolates was 15 (2 from EOS and 13 from LOS). In EOS, the isolated organism was Klebsiella. In LOS, the prevalent isolates were CoNS (7/13), While Klebsiella isolates were (6/13), and Enterococci isolates were (2/13) (Table 2).

A statistically high significant difference was found between the patients' and control groups in this study regarding the expression of CD64 on the surface of neutrophils (p<0.01), while a non-significant difference was detected between both groups regarding the expression of TLR-2 on the surface of monocytes (Table 3).

Studying the performance of the studied diagnostic markers and their combinations in terms of sensitivity, specificity, positive and negative predictive values, we found thatthe combined detection of CRP and TLR-2 showed sensitivity (100%), specificity (50%), positive predictive values (75%), and negative predictive values (100%) (Table 4 and figure 2).

Table (1): Demographic data of the studied patients

	Patients group No=25	
Age\days: Mean ± SD	13.32 ± 7.02	
Sex:	No	%
Male	14	56%
Female	11	44%
Risk factors[no (%)]:		
- Low birth weight ≤ 2500	14 (56%)	
-Prematurity	13 (52%)	
-Maternal risk factor	6 (24%)	
Distribution according to onset of disease[no (%)]:		
EOS	6 (24%)	
LOS	19 (76%)	
CBC & CRP [Mean ± (SD)]:		
WBCs **{(4.5 -10) ×10^3\ dl}**	15.90 ± 8.56	
ANC**(4.5 cells \ mm^3)**	8.07 ± 4.87	

I\T ratio**(≤ 0.12)**	0.21 ± 0.12
Platelets **{(150 - 400)×10^3\ mm^3}**	230.32 ± 158.27
Hb **(14 - 20 gm \ dl)**	13.41 ± 1.94
CRP **(Less than 10 mg \ L)**	25.16 ± 22.85
Blood culture results {No.(%)}	
Culture positive	15 (60%)
Culture negative	10 (40%)

EOS: early onset sepsis
LOS: late onset sepsis
CBC: complete blood count.
WBC: white blood cells.
ANC: absolute neutrophil count.
CRP: C-reactive protein.
I/T ratio: immature/total neutrophil ratio.
Hb: hemoglobin.

Table (2): Isolated organisms among cases of EOS and LOS

Type of organism	Type of neonatal sepsis					
	EOS (n=2)		LOS (n=13)		Total (n=15)	
	No	%	No	%	No	%
CoNS	0	0	7	53.8	7	46.6
Klebsiella	2	100	4	30.7	6	40
Enterococci	0	0	2	15.3	2	13.3

EOS: early onset sepsis.
LOS: late onset sepsis.
CoNS: coagulase negative staphylococci.

Table (3): Comparison between the mean percentage of cells expressing CD64 and TLR-2 detected by flowcytometry in studied patients and control groups

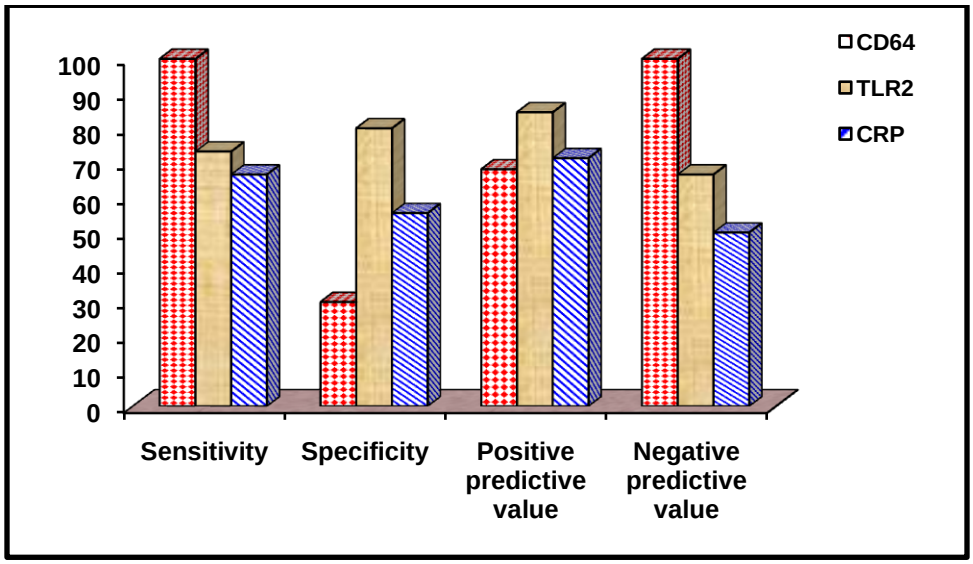
Surface markers	The studied groups		p value
	Cases	Control	
CD64 on neutrophils % **Mean ± SD**	58.45±32.54	23.56±23.57	<0.01*
TLR-2 on monocytes % **Mean ± SD**	91.18±9.87	94.09±5.63	> 0.05

p <0.01: highly significant difference

p> 0.05: non-significant difference

Table (4): Performance of the studied diagnostic markers and their combinations for the diagnosis of neonatal sepsis

Parameter	Sensitivity	Specificity	Positive predictive value	Negative predictive value
CD64	100%	30%	68.2%	100%
TLR2	73.3%	80%	84.6%	66.7%
CRP	66.7%	55.6%	71.4%	50%
CD64+TLR2	100%	10%	62.5%	100%
CRP + CD64	100%	20%	65.2%	100%
CRP + TLR2	**100%**	**50%**	**75%**	**100%**



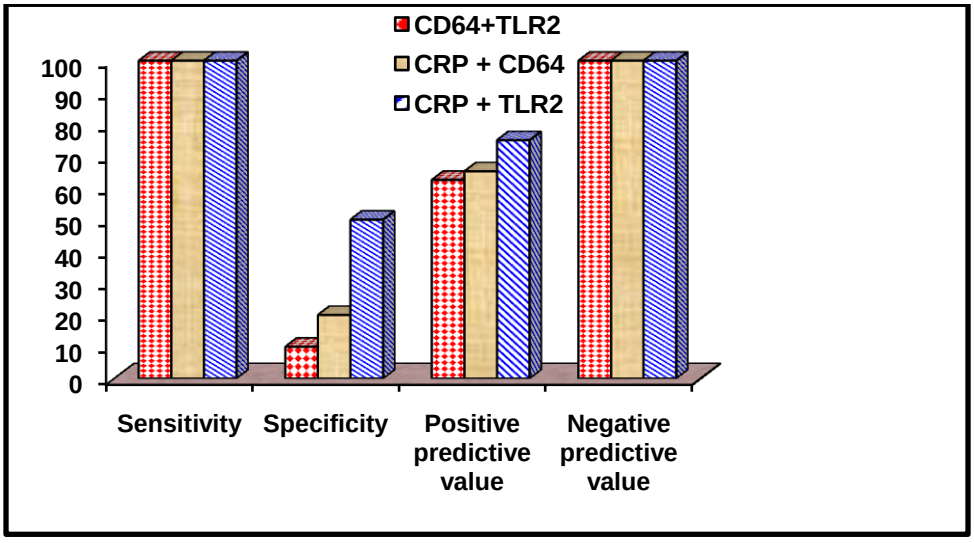


Figure (2): The validity of diagnostic markers and their combinations in the diagnosis of neonatal sepsis

DISCUSSION

Neonatal sepsis remains a global health problem owing to its significant contribution to morbidity and mortality. Its early diagnosis presents a clinical dilemma because of the variable and non-specific clinical presentation **(Dhlamini et al., 2013).**

The diagnosis of neonatal sepsis is a challenge, as there is no single reliable test for its early conformation or exclusion. Blood culture is the most reliable method for detection of bacterial infections. However, the sensitivity of this method is low and using it as a gold standard in the diagnosis of bacteraemia is fraught with difficulties including delay in final culture results (2 - 4 days), negative culture results in patients exposed to antibiotics, and technique of specimen collection. Despite being widely known as lacking specificity and sensitivity, the white blood cell count (WBC), platelet count and white cell differential count are still used for the screening of neonates with sepsis **(Ng and Lam, 2006; Hoffmann, 2009)**.

An early biomarker of bacterial infection with high diagnostic sensitivity and specificity would be a valuable tool for therapeutic decision making, enabling unnecessary use of antibiotics in patients clinically suspected to have infection to be avoided **(Cid et al., 2010; Chan and Gu, 2011)**.

The aim of this study was to assess the utility of detection of CD64 antigen on neutrophils and TLR-2 on monocytes by flow cytometry as early markers for diagnosis of neonatal sepsis compared to conventional blood culture.

In the present study, hematological laboratory indices are estimated among cases. The results reveal that total leukocytic count and the I\T ratio of neutrophils are higher in patients compared to normal standard ranges,while the mean hemoglobin levels were low. There isa mild non-significant decrease in platelet counts.

These results were in agreement with **Bhandari et al. (2008) and Mondal et al. (2012)** who found that the hematologic profiles of neonates with septicemia were characterized by higher white blood cell count, high immature/total neutrophil ratio, lower platelet count and hemoglobin level.

On the other hand, **Sucilathangam et al. (2012)** reported that total WBCs count and Hb level were normal in (85%) of cases.

Anwer et al. (2000) reported that thrombocytopenia with counts less than 100,000 may occur in neonatal sepsis and generally observed after sepsis has been diagnosed and usually lasts one week. Although it can last as long as 3 weeks, only 10-60% of infants with sepsis have thrombocytopenia because of the appearance of newly formed platelets.

I\T neutrophil ratio is believed by many as a single most helpful test available for diagnosing neonatal sepsis **(Polin et al., 2005; Bhandari et al., 2008)**. A study by **Buch et al., (2011)** concluded that the most useful individual test for confirming and excluding neonatal sepsis was (I\T) ratio. When diagnosing sepsis, the elevated (I\T) ratio should be used in combination with other signs. This is attributed to the release of neutrophils from bone narrow in response to infection, with increasing number of immature cells entering the blood stream and producing a differential cell count with a shift to the left **(Linda, 2002)**.

On the other hand, **Aly et al., (2012)** reported that abnormal neutrophil counts taken at the time of symptoms onset are only observed in two thirds of infants, So the neutrophil count does not provide an adequate confirmation of sepsis.

Chirico and Loda, (2011) observed that hematological counts and ratios showed a limited accuracy with wide range of sensitivity (17- 90%) and specificity (31-100%), due to the relatively long period necessary to give positive results and the significant influence of non-specific factors. However, the (I/T) ratio of > 0.2 may reach a sensitivity of 90% and negative predictive value of 98%

Variations in the results shown by different studies may be due to differences in blood sampling time, severity of infection, the age of neonates and reduced sensitivity of these tests in the first week of life **(Aly et al., 2012)**.

Newman et al., (2010) demonstrated that the diagnostic accuracy of the WBC, and (I\T) ratio increased from 0 hour to 4 hours of life. These data suggested that age-specific ratios are better than dichotomous interpretation of the CBC based on fixed 'normal' ranges, and provide better information about the risk of EOS. Future refinements of the CBC may include the development of computerized algorithms, which will modify variables such as postnatal age to compute the probability of sepsis.

In the present study, blood cultures were done for all cases and accordingly patients were classified into two groups: blood culture positive cases (culture proven sepsis), their number were 15 / 25 (60%) and blood culture negative cases (culture unproven sepsis), and their number were 10 / 25 (40%). Isolates of EOS cases were (n=2) which were Klebsiella (100%) while that of LOS cases (n= 13) were; CoNS 7 (53.8%), Klebsiella 4 (30.7%) and Enterococci 2 (15.3%).

These results were consistent with **Kaseb et al. (2004)** who found that gram-positive bacteria as staphylococcus were isolated in (50%) of the cases, while Klebsiella was found in (26.7%).

Also**Marchant et al., (2013)** found that gram-positive organisms accounted for the majority of neonatal sepsis cases (up to 70%) while sepsis due to gram-negative organisms accounted for (15 to 20%).

On the contrary, **Aletayeb et al., (2011) and Shah et al., (2012)** reported that the percentage of Gram -ve isolates from sepsis cases were (92.8%) and (52%) respectively.

Ballot et al., (2012) found that Gram-positive infections predominated in EOS, with Streptococcus agalactiae being the most common pathogen.

Li et al., (2013) also reported that Coagulase-negative Staphylococcus (CoNS) was the major Gram-positive bacteria in late onset sepsis (LOS) (54.4%). On the other hand, **Muhammad et al., (2010)** found that Klebisella and Moraxella were the most common isolates of LOS.

This variability in the etiology between EOS and LOS can be explained by the fact that EOS is conventionally regarded as maternally-acquired, with causative organisms, such as Gram negative organisms which are usually found in the maternal genital tract, whereas LOS is considered environmental in origin-either hospital or community acquired **(Aly et al., 2012)**.

Intervention procedures may affect the distribution of microorganisms. As regard EOS, after introduction of intrapartum GBS prophylaxis, the rate of this infection decreased and allowed Gram negative predominance to occur, while predominance of CoNS in LOS is attributed to the poor infection control attitude in hospitals especially in developing countries **(Stefanovic, 2011)**.

In this study, CRP showed a sensitivity of (66.7%), specificity (55.6%), positive predictive value (71.4%), and negative predictive value (50%). These values demonstrate that it cannot be used as a single marker for the diagnosis of neonatal sepsis.

CRP has been evaluated by many workers but the lack of specificity was the main disadvantage, **Schmit and Vincent, (2008)** reported that sensitivity of CRP was (77%), and specificity was (67%). Also **Sucilathangam et al., (2012)** reported that sensitivity and specificity of CRP were (50%) and (69%) respectively in culture proven cases.

Rajendra et al., (2012) found that the sensitivity, specificity, PPV and NPV of CRP test in culture proven cases were (87.37%), (71.43%), (73.45%) and (86.21%) respectively.

The common causes of false-positive CRP values in neonates are surgery, immunizations, and severe viral infections such as herpes and rotavirus **(Weitkamp and Aschner, 2005)**.

The qualitative assay of CRP does not offer significant advantages over the leukocyte indices. On the other hand, quantitative CRP values, particularly when repeated, are highly specific and have good sensitivity. In addition, serial measurements can be helpful in monitoring the response to treatment. Two serial CRP values <1 mg/dl, excludes the sepsis soon after birth and carry a 99% negative predictive value. In spite of the reduced early sensitivity, CRP still remains the preferred index in most NICUs **(Chirico and Loda, 2011)**.

Flowcytometric analysis has the advantage over conventional immunological assay methods being able to localize the activated markers to a specific cell type. In newborns, cell surface antigens have been studied in connection with congenital sepsis, EOS and LOS. Neutrophil CD11b and CD 64 have been found to be promising markers for diagnosis of early and late infections respectively **(Ng, 2004).**

The results of this study show a statistically significant difference between patients and control groups regarding percentage of expression of CD64 on neutrophils. In patients group, the mean percentage of expression was (58.45±32.54), while in control group, it was (23.56±23.57).

These results are consistent with others as **Khalifa et al., (2007)** who reported that the mean percentage of expression of CD64 on neutrophils in patients group was (82.3±12.3) compared to (8.09 ±3.7) in the control group. Also **El-Mazary et al., (2010)** reported that the mean expression of CD64 in the patient group was higher than that of control group.

Using the blood culture for diagnosis of infected neonates as the gold standard technique, our results showed that neutrophil CD64 has sensitivity, specificity, PPV and NPV of 100%, 30%, 68.2% and 100% respectively.

These results of CD64 expression on neutrophils go with others, as **Choo et al., (2012)** who reported sensitivity of 91% and specificity of 83%. **Faix, (2013)** also demonstrated that sensitivity and specificity of CD64 expression were 75% and 77% respectively. **Jia et al., (2013)** reported sensitivity of 78%, specificity of 81%, and **Streimish et al., (2014)** demonstrated that sensitivity was 78% and specificity was 59%.

CD64 index has some general favorable characteristics as it does not differ with age, because its expression only occurs upon cell activation and is stable for more than 30 hours at room temperature. In addition to accuracy, the laboratory test for CD64 is rapid (<60 minutes) with the use of flow cytometry and requires minimal blood volume (<100 μL) which is a real advantage in neonates **(Choo et al., 2012)**.

Several published studies have indicated that neutrophil CD64 is an ideal candidate for evaluation as a more sensitive and specific marker of infection **(Bhandari et al., 2008; Hoffmann, 2009).**

Another surface marker that has been studied is TLR-2 expression on monocytes which is a component of the innate immune response, and is involved in the recognition of PAMPs, and activates most of the antimicrobial mechanisms that are usually analyzed as diagnosis markers e.g. CRP,procalcitonin, cytokines, etc.**(Ramírez et al., 2012)**.

In the present study the difference between patients and control groups regarding the mean percentage of expression of TLR-2 on monocytes is statistically insignificant. In patients group, the mean percentage of expression is (91.18±9.87), while in control group, it is (94.09±5.63).

Using the blood culture for diagnosis of infected neonates as the gold standard, monocyte TLR-2 parameters show sensitivity, specificity, PPV and NPV 73.3%, 80%, 84.6% and 66.7% respectively.

These results were consistent with **Ramirez et al., (2012)** who found that the TLR expression values were low. They explained these results due to the opportune administration of therapy, which was sufficient to control

microorganism proliferation without the activation of an immune response. Alternatively, the effect may be due to the under development of the immune system in a premature infant.

On the other hand, **Viemann et al. (2005)** who examined the TLR-2 and TLR-4 expression on granulocytes and monocytes reported that there was a TLR-2 significant upregulation in the group of neonates with septicemia compared to healthy neonates, based on CRP, IL-6 and IL-8 results. Also **Zhang et al. (2010)** reported that monocyte and granulocyte TLR-2 expression was higher in the Gram positive bacterial group than Gram negative bacterial group and control group.

This study shows that the combination of CRP and TLR2 has the highest sensitivity, specificity, positive and negative predictive values (100%, 50%, 75%, 100%).

Recent investigations have focused on the combination of markers ensuring greater diagnostic accuracy. **Ng et al., (2002)** found that the use of CD64 in combination with other diagnostic markers such as IL-6 or CRP improved the sensitivity and negative predicative value to 100% for late onset neonatal infection.

For early onset neonatal infection, **Ng, (2004)** found that the addition of CRP to CD64 enhanced the sensitivity and negative predicative value to 97% and 98% respectively. **Bhandari et al., (2008)** reported that the combination of neutrophil CD64 and ANC has improved the sensitivity and negative predictive value to 95% and 93% respectively. **Genel et al., (2012)** demonstrated that the combination of CRP and neutrophil CD64 have high sensitivity for detecting neonatal infection. Yunanto et al. (2012) found that TLR-2 and TLR-4 combination is statistically significant between the control group (2.85 ± 2.08) and the case group (7.17 ± 5.06).

Conclusion:

Flowcytometric assessment of neutrophil CD64 and monocyte TLR-2 was able in this study to differentiate between neonates with neonatal sepsis from neonates free from sepsis. High NPV of CD64 indicates its role in ruling out bacterial sepsis, while specificity of TLR-2 indicates its role in detecting neonates free from sepsis. Thus, they can be considered good markers for early diagnosis of neonatal sepsis.Combination of the studied markers such as CRP and TLR-2 was associated with higher sensitivity, specificity and NPV. However, blood culture remains irreplaceable in diagnosis of neonatal sepsis as it is the gold standard test and antimicrobial sensitivity tests are indispensable clue for proper choice of antibiotic therapy.

Recommendations:

Further studies are needed to measure the effectiveness of each leucocyte surface marker as a diagnostic tool and highlight more clearly the role of these markers in neonatal sepsis.

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