Annals of Neonatology

OPEN ACCESS
ISSN: 2636-3596

Original Article

Serum Cortisol Level and Monocyte HLA-DR Expression in Late Onset Neonatal Sepsis: A Case-Control Study

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DOI: 10.21608/anj.2025.300550.1093

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Abstract

Background: Neonatal sepsis remains a substantial cause of morbidity and mortality in the nursery setting. Neonatal sepsis is challenging to diagnose because its manifestations are non-specific (e.g. respiratory distress, hypotension, and apnea) which could be presented in non-infectious conditions.

Objectives: Our aim was to study the value of serum cortisol level and expression of HLA-DR on monocytes in early diagnosis of late onset neonatal sepsis .

Methods: This case-control study conducted on 60 neonates diagnosed as late onset neonatal sepsis divided into 2 groups: Definite sepsis group: included 30 neonates and Probable Sepsis group: included 30 neonates in addition to 30 apparently healthy age-matched control group. All groups subjected to complete blood count, blood culture, C-reactive protein, serum cortisol and Flow cytometric assessment of human leukocyte antigen–DR (HLA-DR) on monocytes.

Results: We founded that the definite sepsis group had statistically significant higher values of cortisol and CRP levels and lower values of expression of HLA-DR on monocytes than probable sepsis (p-value = 0.0001 for all) Moreover, the definite and the probable sepsis groups had significantly higher cortisol and CRP levels and lower values of expression of HLA-DR on monocytes than controls (p-value = 0.0001 for all).

Conclusion: Cortisol level and HLA-DR expression on monocytes are reliable indices for early diagnosis of late onset neonatal sepsis, but alone cannot be dependable for accurate diagnosis.

Keywords: Neonatal sepsis; Cortisol; Monocyte Expression, HLA-DR; Flow cytometry

Introduction

Neonatal sepsis is a clinical syndrome caused by pathogenic bacteria which can invade the blood circulation and attack the reproduction system of neonates during the 1st month of life and cause hemodynamic changes and other systemic symptoms of infection [1].

Neonatal sepsis may result in tissue damage and organ failure, which is a reason for neurocognitive sequelae and neonatal mortality [2].

According to the age of onset, neonatal sepsis is divided into early-onset sepsis and late-onset sepsis.

Early-onset sepsis (EOS), diagnosed ≤ 72 hours after birth, is mostly related to antenatal and perinatal factors including prolonged rupture of amniotic membranes, maternal chorioamnionitis and maternal colonization with group B streptococcus (GBS).

Late-onset sepsis (LOS), diagnosed >72 hours after birth, is primarily hospital acquired with the peak incidence reported to be between the 10th & 22nd day of life [3].

Major risk factors for LOS include the use of indwelling vascular catheters , prolonged mechanical ventilation and necrotizing enterocolitis. Patients with late-onset sepsis have poor prognosis with a prolonged hospital stay, and 18% of mortality in neonates caused by late-onset sepsis [5]. Early symptoms of late-onset sepsis are often atypical, and septic shock often occurs due to delayed diagnosis [6].

The early diagnosis of sepsis will ensure a prompt treatment, thus reducing organ failure and mortality. However, there are challenges in the early diagnosis, for example, blood culture which is the gold standard test needs time 2 to 4 days to confirm the diagnosis, and possibility of having a negative result in neonates who were exposed to antibiotics in utero (4, 5).

The unnecessary administration of antibiotics in non-septic patients increases the economic burdens and

exposes the infants to unnecessary treatment thus, there is a rising need for a diagnostic indicator for timely sepsis diagnosis [6].

During bacterial infections, the interacting leukocytes increase the expression of cell adhesion molecules; these cell surface antigens are considered as new markers in the diagnosis of sepsis and identified easily by flow cytometry, a technique that robustly progressed [7].

HLA-DR is on the surface of monocyte/macrophages, dendritic cells, and B cells and plays a crucial role in adaptive immune response. A decreased expression of monocytic human leukocyte antigen–DR (mHLA-DR) molecules has been associated with immunoparalysis, which is an anti-inflammatory immune response that occurs in sepsis [8, 9].

Endogenous cortisol is one of the main components of the anti-inflammatory response induced by the central nervous system during septic shock [10]. Its down-modulating effect on monocyte HLA-DR expression has been described in other clinical settings and in vitro observations [11]. Basal plasma cortisol levels are higher in the patients who have the highest risk of mortality. Paradoxically, a beneficial effect of supplementation by low doses of corticosteroids has been demonstrated in catecholamine-dependent septic shock [12].

Patients and Methods

Patients

This study was conducted on 60 neonates admitted to the Neonatal Intensive Care Unit (NICU) at Minia University Children Hospital in addition to 30 apparently healthy neonates as control group in the period from (October 2023 to April 2024). included preterm and full-term neonates aged from 4- 28 days, diagnosed as having late onset neonatal sepsis (according to clinical signs of sepsis and laboratory investigations). The studied neonates were grouped as follow: Definite sepsis group (group 1): included 30 neonates

with clinical signs of sepsis and positive blood culture. Probable Sepsis group (group 2): included 30 neonates with clinical signs of sepsis, two screening parameters positive and sterile blood culture, and Control group (group 3): included 30 apparently healthy neonates, gestational ages, postnatal ages and sex matched with the previous two groups and normal laboratory parameters and clinically free of signs of neonatal sepsis. We excluded from this study: neonates aged ≤ 72 hours or with congenital anomalies or neonates suffering from hypoxic-ischemic encephalopathy or inborn errors of metabolism. All included neonates were subjected to history taking (prenatal, natal and postnatal history), with stress on time of onset of sepsis and risk factors related to sepsis and clinical examination, signs of neonatal sepsis Including : lethargy, fever, tachycardia, abdominal distension, increased prefeed aspirate, chest retraction and grunting , In addition to sick looking, poor suckling, hypothermia, apnea, tachypnea and bradycardia (13).

Samples collection

Five mls of venous blood were collected in early morning under complete aseptic precaution: 1 ml of blood was inoculated into pediatric Bactec bottles For aerobic blood cultures (only for sepsis groups) ,2 ml of blood in EDTA tube for CBC and Flow cytometric assessment of HLA-DR on monocytes and 2 ml of blood into plain tube were collected and allowed to clot then centrifuged and analyzed for serum C-Reactive protein (CRP) and serum cortisol.

Blood samples from neonates suspected of sepsis were withdrawn at the time of clinical diagnosis of sepsis, before initiation of antibiotic therapy.

Laboratory methods

Blood culture: using (BD™ BACTEC™ FX40 Automated Blood Culture System, Becton Dickinson, USA) and subcultures done for positive cases to identify the causative organisms [identification and antibiotic sensitivity

test done by (VITEK-2, bioMérieux, France)].

Complete blood count of all patients were evaluated by automated cell counter (CELLTAC G, NIHON KOHDEN CORPORATION ″Automated Hematology Analyzer ″, Japan). Differential leucocytic count was confirmed by a microscopic examination of Lieshman-stained blood film, **CRP** was performed using GENRUI, biotech Inc, kinetic assay, China. **serum cortisol** determined by ELISA (Diagnostics Biochem Canada Inc., Canada (Catalog No. CAN-C-270). Flow cytometric assessment of **HLA-DR** was performed within 24 h of sample collection. In brief, Staining procedure: For each sample, 2 tubes were prepared labeled 1&2(1 for test tube and 2 for isotypic control).100 µL of blood sample was added to the tubes. Ten µL of anti HLA-DR FITC conjugated antibody was also added (only to the test tube). Both tubes were incubated for 15-20 minutes at room temperature in the dark, then washed by PBS to remove any unbound antibodies Followed by red cell lysis using 2 ml of lysing solution then incubated for 10 minutes at room temperature in the dark then centrifuged for 5 minutes, supernatant was discarded and 2 ml of Phosphate buffered saline were added. Wash by PBS was repeated twice then the cells were re-suspended in 300 µL of PBS for final flow cytometric analysis. Analysis was carried out using a (BD FACS canto II, U.SA). Data processing was carried out with the Diva software.

Ethical consent

The study was explained in details to the parents of the participant neonates and written consents were taken from them. It was performed according to the Declaration of Helsinki 1975, as revised in 2008 and approved by the Institutional Review Board and Medical Ethics Committee of Minia University (Approval number:864:8:2023, Date of approval: 14 august 2023).

Statistical analysis

By using the Statistical Package for the Social Sciences (SPSS) program for Windows, version 22. Quantitative results were presented as mean± SD while qualitative data were presented by frequency distribution as percent (%). Student's sample t test, ANOVA and chi square were used for comparison and Z test was used to compare proportions. Receiver operating characteristic (ROC) curve analysis was performed to determine: the optimal cutoff values, the detective performance of different studied markers and scores, and their sensitivities and specificities for the detection of late onset neonatal sepsis. less than 0.05 was used as a cutoff point for all significant tests.

Results

In this study, the Definite Sepsis group were 14 males and 16 females, they had a mean gestational age of 37±2.5 weeks and a mean postnatal age of 12.1±4.3 days, and the Probable Sepsis group were 17 males and 13 females, with a mean gestational age of 37.6±2.4 weeks and a mean postnatal age of 10.4±4.08 days. While the Control group were 16 males and 14 females, their mean gestational age was 38.4 ±1.3 weeks and their mean postnatal age was 8.5±4.5 days. There were no statistically significant differences between the three groups regarding demographic data (Table 1).

We compared the clinical signs in neonates with probable and definite sepsis, and there was only significant difference between the two groups regarding blood culture results but no statistically significant difference in clinical signs.

The isolated organisms were Klebsiella Pneumoniae from 14 neonates (46.6%) and Escherichia coli from 6 neonates (20%), staphylococcus pneumoniae and staphylococcus aureus each from 3 neonates, (10%) for each organism, Methicillin-resistant Staphylococcus aureus (MRSA) from 2 neonates (6.6%) and Pseudomonas aeruginosa was

isolated from only one neonate (3.3%). Neonates with negative blood cultures (n = 30) were considered the probable sepsis group (clinical sepsis) (Table 2).

Regarding hemoglobin level and platelets count, there was statistically significant decrease in hemoglobin level and platelets count in the definite sepsis group when compared to the probable sepsis group and control group and statistically significant decrease in hemoglobin level and platelets count in the probable sepsis group when compared to the control group (P value= 0.0001* for all) and (P value = 0.0001* for all) respectively. While total leucocyte count (TLC), there was statistically significant increase in total leucocytic count in the definite sepsis group when compared to the probable sepsis group and the control group and statistical significant increase in total leucocytic count in the probable sepsis group when compared to the control group (P value = 0.0001* for all) (Table 3).

Concerning blood cells surface markers evaluated by the flow cytometry, mHLA-DR percent was statistically significant decreased in definite sepsis group when compared to probable sepsis group and control group and statistical significant decrease in mHLA-DR percent in probable sepsis group when compared to control group (P-value = 0.0001* for all) , while statistically significant increase in cortisol level and CRP level in definite sepsis group when compared to probable sepsis group and control group and statistical significant increase in cortisol level and CRP level in probable sepsis group when compared to control group (P value = 0.0001* for all) and (P-value = 0.0001* for all) respectively (Table 4).

ROC curve analysis for detection of sepsis for comparison of definite to controls revealed that mHLADR at a cut-off value of ≤ 23.5 had AUC (0.99±0.009) with highest sensitivity (96.7%) and specificity (90%), (P value

= 0.0001*), while cortisol at a cut-off value of ≥25 had AUC (1±0.0) with highest both sensitivity and specificity (100%) for both, (P value = 0.0001*) (Table 5 and Figures 1 & 2).

ROC curve analysis for detection of sepsis for comparison of probable to controls revealed that mHLA-DR at a cut-off value of ≤ 42 had AUC (0.83±0.05) with highest sensitivity (80%) and specificity (73.3%%), P 0.0001*), while cortisol at a cut-off value of ≥ 22 had AUC (1±0.0) with highest sensitivity (100%) and specificity (100%), (P-0.0001*). (Table (6) and Figure (3 and 4).

Table (1): Demographic data of included neonates

Variables	**Definite sepsis group (n = 30)**	**Probable sepsis group (n = 30)**	**Control group (n = 30)**	**p-value**
Gestational age (weeks)				
Range	34 - 39	35 – 40	36 - 39	0.326
Mean±SD	37±2.5	37.6±2.4	38.4 ±1.3	
Postnatal age (days)				0.326
Range	8 – 17	6 – 15	5– 14	
Mean±SD	12.1±4.3	10.4±4.08	8.5±4.5	
Sex:				
Male N (%)	14 (46.6%)	17(56.6%)	16 (53.3%)	0.372
Female N (%)	16 (53.3%)	13 (43.3%)	14 (46.6%)	

*: Significant difference at P value < 0.05

Table (2): Clinical signs of the two sepsis groups and blood culture results of the definite sepsis group

Variables	Definite sepsis group (n = 30) n (%)	Probable sepsis group (n = 30) n (%)	p-value
Tachycardia	8 (26.6 %)	6 (20%)	0.2
Fever	8 (26.6 %)	11 (36.6%)	0.4
Increased prefeed aspirate	13 (43.3 %)	11 (36.6%)	0.3
Abdominal distension	10 (33.3%)	10 (33.3 %)	0.5
Grunting	10 (33.3%)	6(20%)	0.15
Chest retraction	19 (63.3%)	20 (66.6%)	0.4
Hypothermia	11 (36.6%)	5 (16.6%)	0.13
Poor suckling	25 (83.3%)	18 (60%)	0.07
Bradycardia	3 (10%)	2 (6.6%)	0.24
Tachypnea	14 (46.6%)	11(36.6%)	0.22
Apnea	15 (50%)	10 (33.3%)	0.11
Blood culture:			
- Klebsiella	14 (46.6%)	0 (0%)	< 0.0001*
- Escherichia coli	6 (20%)	0 (0%)	0.009*
- Streptococcus pneumoniae	3 (10%)	0 (0%)	0.07
- Staphylococcus aureus	3 (10%)	0 (0%)	0.07
- MRSA	2 (6.6%)	0 (0%)	0.1
- Pseudomonas aeruginosa	1 (3.3%)	0 (0%)	0.3

*: Significant difference at P value < 0.05

Table (3): Comparison of the different studied groups as regard hematological parameters

Data		Definite No=30	Probable No=30	Controls No=30	P	p1	p2	p3
Hemoglobin level(g/dl)	**Range**	7.3-15.5	9.7-17	12.3-16.6	0.0001*	0.0001*	0.0001*	0.0001*
	Mean±SD	10.2±2.2	11.9±1.9	14.5±1.1				
Total leucocytic count (x10^3 /µl)	**Range**	6-33.6	5-15	5-11.1	0.0001*	0.0001*	0.0001*	0.0001*
	Mean±SD	18.6±7.05	10.6±2.4	8.7±1.3				
Platelet count (x10^3 /µl)	**Range**	50-220	90-298	210-399	0.0001*	0.0001*	0.0001*	0.0001*
	Mean±SD	133.7±39.1	196.1±49.8	304.8±52.				

P = Between all groups, P1=Definite vs controls, p2= probable vs controls, p3= definite vs probable.
*: Significant difference at P value < 0.05
**: Highly significant level at P value < 0.001

Table (4): Comparison of the different studied groups as regard mHLA-DR, serum cortisol and CRP

Data		Definite No=30	Probable No=30	Controls No=30	P	p1	p2	p3
mHLADR (%)	**Range**	5-25	10-58	20-76	0.0001*	0.0001*	0.0001*	0.0001*
	Mean±SD	14.2±6.3	33.5±12.9	53.9±15.8				
Cortisol (ug/ dl)	**Range**	30-51	24-43	9-20	0.0001*	0.0001*	0.0001*	0.0001*
	Mean±SD	44.2±5.1	32.6±5.3	14.1±3.6				
CRP (mg/ l)	**Range**	25-75	9-35	1.8-40	0.0001*	0.0001*	0.0001*	0.0001*
	Mean±SD	54.3±15.4	19.8±7.02	4.8±6.7				

P = Between all groups, P1, Definite vs controls, p2= probable vs controls, p3= definite vs probable.
mHLADR: Monocytic human leukocyte antigen–DR ., CRP: C reactive protein

Table (5): AUC, Sensitivity and Specificity of mHLA-DR and Cortisol Level for comparison of definite to controls

Data	AUC	Cutoff	Sensitivity	Specificity	p-value
mHLA-DR	0.99±0.009	≤ 23.5	96.7%	90%	0.0001*
Cortisol	1±0.0	≥ 25	100%	100%	0.0001*

Table (6): AUC, Sensitivity and Specificity of mHLA-DR and Cortisol Level for comparison of probable to controls

Data	AUC	Cutoff	Sensitivity	Specificity	p-value
mHLA-DR	0.83±0.05	≤ 42	80%	73.3%	0.0001*
Cortisol	1±0.0	≥ 22	100%	100%	0.0001*

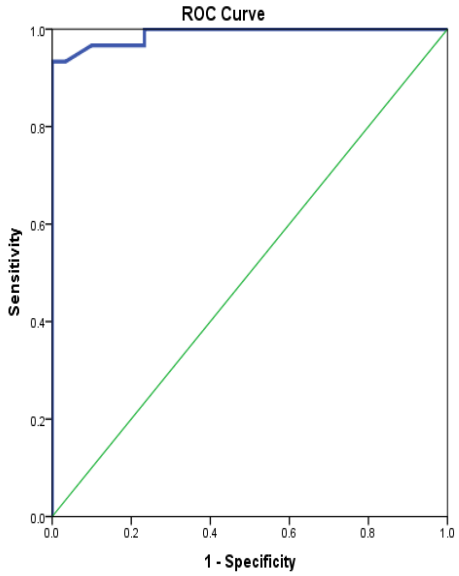

Figure (1): ROC Curve analysis of mHLA-DR for comparison of definite to controls

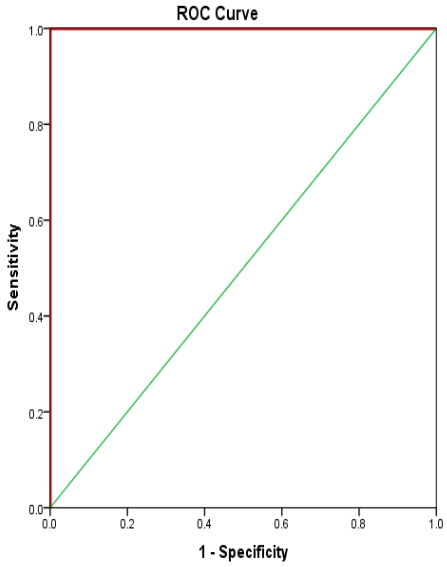

Figure (2): ROC Curve analysis of Cortisol Level for comparison of definite to controls

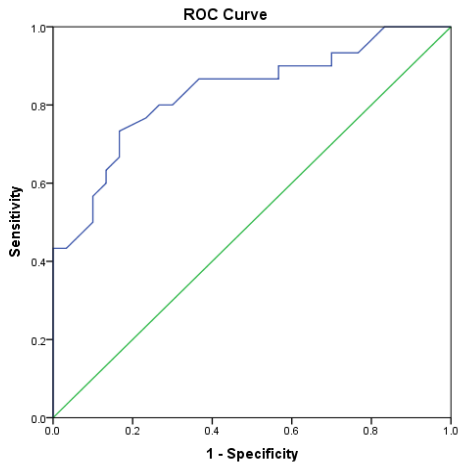

Figure (3): ROC Curve analysis of mHLA-DR for comparison of probable to controls

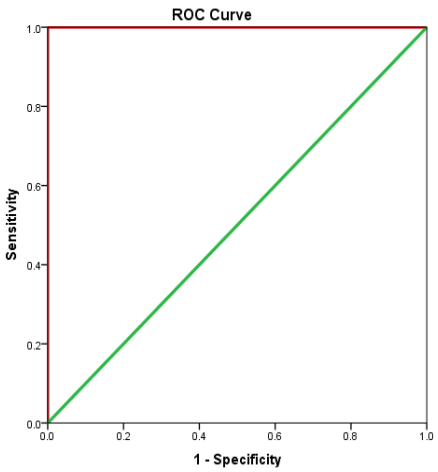

Figure (4): ROC Curve analysis of Cortisol Level for comparison of definite to controls

Discussion

Diagnosis of neonatal sepsis remains a challenge. The signs of sepsis, the frequent sampling. The time taken for blood culture results and the optimal timing of antibiotic treatment make it essential to find the best biomarker to aid the diagnosis of sepsis. In this study, neonates were grouped into three groups: neonates with definite sepsis who had positive blood culture, neonates with probable sepsis due to the presence of

signs of sepsis but negative blood cultures, and healthy neonates (control group). Pro-inflammatory cytokines released in response to microorganisms invasion induce production of proteins of acute-phase response in the liver including CRP, which plays important role in the humoral response to bacterial invasion [14].We also showed that newborns with clinically confirmed sepsis had markedly elevated CRP levels, many researches showed higher level of CRP in neonatal sepsis and used it as a tool to distinguish between healthy newborns and those with definite or probable sepsis [15, 16]. Also, it was significantly higher in definite sepsis group than probable sepsis group as the magnitude of the CRP response to sepsis was reported to depend also on the underlying pathogen [14], also the CRP concentration can reflect the presence and the severity of sepsis [8].

However, as its concentrations increase slowly at first, the sensitivity is only 60% at the time of sepsis and serial measurements are required to improve the sensitivity, and it is also not specific enough as its concentrations can be elevated in other conditions such as tissue necrosis, recent vaccination, surgery and meconium aspiration [7, 14].

Regarding hematological parameters measured in our study; septic group had significantly lower platelet count compared to the non-septic group. In agreement with our results Omran et al., (2021), who found a statistically significant lower platelet count among septic and non-septic groups [17]. Contrary to our results Tosson et al., (2021), didn't find a significant difference between septic group and non-septic group regarding platelet count with (P=0.47) [18]. Also, TLC was significantly higher in septic group than in non-septic group, agreeing with Mubaraki et al., (2023) who found that TLC was significantly higher in septic group [19]. In disagreement with our results, both Omran et al., (2021) and

Tosson et al., (2021), as they did not find a statistically significant difference between septic group and non-septic group [17, 18]. Blood culture is still the gold standard for the diagnosis of bacterial sepsis; however, its results may take much time, because of expected low bacteremia and early antibiotic therapy prior to blood culture withdrawal. Furthermore, hospital rules and standards recommend that it should be collected in the case of a temperature surge to maximize its yield [20]. In the current study, it was found that 30 neonates representing no growth blood culture. While 30 neonates representing positive blood culture results, The commonest microorganisms revealed from blood culture results were; Klebsiella (46.6%), E.coli (20%), streptococcus pneumoniae (10%), staphylococcus aureus (10%), MRSA (6.6%) and Pseudomonas aeruginosa (3.3%) . The most common isolates were gram-negative bacteria predominantly klebsiella pneumoniae (46.6%). These results were in agreement with Elmashad et al. (2019) [21] and Ramavath et al., (2023) [22]. Contrary to our results, Tosson et al., (2021) [18] had MRSA as the most common organism isolated in the cases. Other investigators such as Hammoud et al., (2017) [23], found that the most common pathogen isolated in LOS was Coagulase-negative staphylococci (CONS). There is a variability of results of infective organisms from NICU to another, between geographical areas and in the same area according to time thus each hospital must adjust their antibiotics accordingly. Neonatal sepsis remains challenging . The early diagnosis of sepsis remains difficult tasks, there is a great need to find new indicators for neonatal sepsis to increase the sensitivity and specificity of both diagnosis and monitoring therapy. However, there is no single biomarker available that differentiates between sepsis and systemic inflammation.

Flow cytometry can play a good role in the diagnosis of sepsis. It requires

minimal volume of blood and results appear rapidly. Treatment can be initiated based on the patients' immune system situation assessed by flow cytometric expression of cell surface markers [24]. In the present study, mHLA-DR had shown a positive significance with the possibility of occurrence of sepsis. A study by Winkler et al.,2017 [25], showed a higher number of monocytes, but with lower expression of HLA-DR in peripheral blood of septic patients. Another study by Genel et al., 2010 [26] found a lower HLA-DR level in septic neonates and found a prognostic value for it in this group of patients. On the contrary, the study by Ng et al., 2006 on neonates with suspected sepsis showed no significant differences in monocyte HLA-DR expression between infected, non-infected, and control groups [27].

Cortisol level was significantly higher in septic groups (definite and probable sepsis) than controls and in definite sepsis group than probable sepsis group, this was in agreement with Das et al., 2002 who found that mean serum cortisol level was higher in neonates with sepsis, this could be explained by that sepsis results in marked centrally driven increase in cortisol production by the adrenal cortex [28]. Also, Bhat et al., 2022 revealed increase cortisol level in neonatal septic shock [29].

The ROC curve for HLA-DR showed an area under the curve of 0.99±0.009 and 0.83±0.05 with high sensitivity and specificity and ROC curve for cortisol showed an area under curve of (1±0.0) and high sensitivity and specificity for definite and probable cases in comparison to controls (P =0.0001*), so it is better if we use Cortisol and HLA-DR together in diagnosis of late onset neonatal sepsis.

Limitation: There are several limitations in our study. For example, studies with larger sample size are needed, serial measurements of cortisol level and monocyte expression of HLA-DR during the disease course and the relation of

them to neonatal mortality and morbidity.

Conclusion:

Monocyte expression of HLA-DR in blood decreased and serum cortisol level is increased in presence of late onset neonatal sepsis and they more increased in definite neonatal sepsis than probable neonatal sepsis and it was more specific and more sensitive than CRP. This can help neonatologists to early diagnose late onset neonatal sepsis which leads to early establishing proper treatment and that will improve the outcome and reduce hospital stay of the neonates.

Data Availability

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Acknowledgements

The authors would like to thank the NICU crew of Minia University Hospital. They were all very helpful and supportive

Author's contributions

All authors participated in the design and planning of the study, preparation and review of the final manuscript. EM, NI and MM participated in data collection and preparation of drafts of the manuscript. NI and MM performed the laboratory work interpretation. EE performed Statistical analysis of results. All authors read and approved the final manuscript.

Funding

We had not received financial support for the study

Conflict of interest

We declared no conflict of interest concerning the study.

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Date received: 24th September 2024, accepted 30th January 2025

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Citation: Nagwa I. Okaily; Ebtihal M. Abdelsamei; Ebtesam E. Hassan; Hend M. Moness; Mahmoud M. Moussa. "Serum Cortisol Level and Monocyte HLA-DR Expression in Late Onset Neonatal Sepsis: A Case-Control Study", *Annals of Neonatology*, 2025, 7(1): 140-158. doi: 10.21608/anj.2025. 300550.1093