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RESEARCH ARTICLE

EVALUATION OF POLYMORPHONUCLEAR LEUCOCYTE ELASTASE LEVEL AS AN EARLY DIAGNOSTIC INDICATOR IN NEONATAL SEPSIS.

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Abstract

Neonatal sepsis is a clinical syndrome of bacterial infection characterized by signs and symptoms of systemic involvement during the first month of life. Sepsis is the most common cause of neonatal mortality**.** Neonatal sepsis is an important but underestimated problem around the world. It is defined as disease affecting newborns ≤ 1 month of age with clinical symptoms and positive blood cultures. Infection is an important cause of morbidity and mortality during the neonatal period, despite the great improvements in intensive neonatal care and the use of extended spectrum antimicrobial agents. Polymorphonuclear (PMN) granulocytes play an important role as primary defence in the inflammatory reactions e.g sepsis and multiple organ dysfunction syndromes . They (PMN) use proteinases to digest the inflammatory mediators and tissue debris. One of these proteinases is PMN elastase. Neutrophil elastase is one of three hematopoietic serine proteases stored in large quantities in neutrophil cytoplasmic azurophilic granules. It acts in combination with reactive oxygen species to help degrade engulfed microorganisms inside phagolysosomes. These proteases are also externalized in an active form during neutrophil activation at inflammatory sites, thus contributing to the regulation of inflammatory and immune responses**.**

Objective: is to determine the sensitivity and specificity of PMN elastase levels for the diagnosis of neonatal sepsis and its use as an early indicator of neonatal sepsis .

Material and Methods:

The study group consisted of forty patients with the diagnosis of sepsis (20 confirmed by +ve blood culture plus 20 clinically suspected and the control group included twenty newborn). Inclusion criteria were formerly sought in our subjects who were diagnosed using the Tollner

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scoring system and based on the clinical observations and laboratory findings. The results of white blood cell and platelet counts, immature/total neutrophil ratio, CRP and PMN elastase values were evaluated in the study groups. Enzyme linked immunoassay methods were used to determine the PMN elastase levels.

Results: The mean PMN elastase level was found to be 1204±791.2 ng/mL in patients with comfirmed neonatal sepsis and 1231±750.05 ng/ml in clinically suspected cases ($p<0.001$) and 90.65 ± 33.65 ng/ml in control roup . When the plasma PMN elastase levels were compared between study groups, the specificity was %92, sensitivity was 96% negative estimation value was 90% and positive estimation value was 94%.

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Introduction:-

Neonatal sepsis remains one of the leading causes of morbidity and mortality both among term and preterm infants. Although advances in neonatal care have improved survival and reduced complications in preterm infants, sepsis still contributes significantly to mortality and morbidity among very-low-birth-weight infants in Neonatal Intensive Care Units ***(Shah and Padbury ,2014).***

According to the World Health Organization, approximately four million neonates die annually with a global neonatal mortality rate of 23/1,000 live births. About a million of these deaths are attributable to neonatal infections **(*Alam* et al,2014).**

The total mortality rate for the proven neonatal sepsis was 51% and 42.9% for EOS and LOS, respectively. ***(Shehab El-Din et al,2015).***

There are two clinical forms of sepsis: early onset neonatal sepsis (EOS), which is clinically manifested within the first 72 hours after birth and in which more than 50% of cases are developed within the first 6 hours of life, and late onset neonatal sepsis (LOS), which occurs after the first 72 hours of life and usually results from nosocomial infection ***(Shane and Stoll , 2014).***

The non-specific symptoms and signs of neonatal sepsis make it difficult to establish an early clinical diagnosis and antibiotic therapy is usually initiated on risk factor or neonatologist suspicion to prompt early treatment as even delay of few hours in initiating treatment can lead to serious morbidity or in some cases, mortality ***(Jan et al, 2013).***

It is important to diagnose neonatal sepsis early and accurately as the unnecessary exposure to antibiotics, with emergence of bacterial resistance will lead to potential poor outcomes in this vulnerable population of neonates. To identify accurately neonates with sepsis, attempts have been made to use physiologic parameters, hematologic indices and cytokine profiles at the time of onset of the suspected sepsis episode **(*Bhandari et al, 2008*). .**

Polymorphonuclear (PMN) granulocytes play an important role as primary defence in the inflammatory reactions e.g sepsis and multiple organ dysfunction syndromes . They (PMN) use proteinases to digest the inflammatory mediators and tissue debris. One of these proteinases is PMN elastase ***(Paysali et al,2013).***

There is evidence suggesting that PMN are involved in the pathogenesis of sepsis and multiple organ dysfunction syndromes . Release of interleukin-8 (IL-8), which is strongly chemotactic for PMN and induces the expression of adhesion molecules on endothelial cells, may encourage activated PMN to adhere to endothelial cells, thereby inducing endothelial damage ***(Lee and Downey, 2001).***

Elastase is considered to be one of the most potent proteolytic enzymes present in azurophilic granules of neutrophils. It plays an important physiological function in degrading phagocytosed substances and facilitating cell migration through vascular walls. Elastase degrades mainly the essential elements of the interstitium (elastin, collagen, proteoglycans) ***(Payasli et al,2013).***

The aim of the study is to determine the sensitivity and specificity of PMN elastase levels for the diagnosis of neonatal sepsis and its use as an early indicator of neonatal sepsis .

Material and Methods:-

This prospective study was performed on newborns who were hospitalized for neonatal sepsis at the Neonatal Intensive Care Unit, Pediatric Department, Alsanta Central Hospital in the period from September 2015 to December 2015 . The protocol was approved by the local ethics committee. Informed written consent was obtained from parents of all the patients.

Forty newborns were diagnosed as sepsis (20 confirmed by positive blood culture, the other 20 negative blood culture but have clinical symptoms and signs of sepsis) based on their Hematological Score and Tollner Score results (Hematological Score ≥3 and Tollner Score ≥10). Clinical signs of sepsis were defined as the presence of three or more of the following categories of clinical signs: apnea, tachypnea (>60/min), respiratory distress, hypotonia, bradycardia (<100/min), tachycardia (>180/min), seizures, change in skin colour and perfusion, irritability, and lethargy. Two or more abnormal values of the sepsis screen were considered as supportive for diagnosis of infection. The control group included twenty neonates who were admitted to the hospital with non-infectious diseases, such as hypoglycemia, indirect hyperbiluribinemia, intrauterine growth retardation, transient tachypnea, without clinical findings of infection.

At time of admission , blood samples for blood culture, routine biochemistry, whole blood count, peripheral blood smear, immature neutrophil: total neutrophil (I/T) ratio, C-reactive protein (CRP) and PMN elastase were taken. Chest radiograph, cerebrospinal fluid culture and urine culture were done whenever clinically indicated.

Complete blood counts were carried out with an automatic counter. Leukocytosis was considered if the white blood cell (WBC) count was more than 25000/mm3 at birth or 30000/mm3 at 12-24h and leukopenia less than 5000/mm3, and thrombocytopenia less than 150000/mm3. By examining peripheral blood smears prepared with Giemsa stain, band forms, myelocytes and metamyelocytes in leukocyte formula evaluated as immature neutrophils and I/T ratio were calculated. Pathologic I/T ratios were greater than 0.2. Specimens of blood were obtained from each infant by a sterile technique and were inoculated into commercially-prepared BD Bactec Peds Aerobic/F vials (Peti-Bact blood culture, Organon Technica). Positive cultures were detected by chemical sensors sensitive to increases in carbon dioxide produced by growth of the organisms. The organisms were then identified based on gram staining and growth on agar media.

Blood samples were obtained on admission, their serum extracted and CRP investigation was made immediately, remaining serum was preserved for PMN elastase evaluation. Blood samples that were obtained from the control group and from neonates with sepsis were centrifuged at 2500 x g for 15 min and the serum portion was preserved by freezing at -20°C for PMN elastase studies.
C-reactive protein level was carried out using the test kit (Cromatest) at 0h of clinical presentation. CRP levels ≤6 mg/L were accepted as normal. Polymorphonuclear elastase levels were measured with an enzymelinked immunosorbent assay (ELISA) with PMN elastase study kits Immundiagnostik AG,Stubenwald-Allee 8a, D 64625 Bensheim.

Statistical analysis:-

The collected data will be analyzed using The Statistical Package for Social Sciences (SPSS) 16 soft ware. Categorical data will be presented as number and percentage. Continuous variable will be expressed as mean±SD.
Comparisons between the study groups were analyzed by the unpaired Student's t test for normally distributed (parametric) data. P value less than 0.05 was considered to be statistically significant. To determine a diagnostic value for PMN elastase and CRP in newborns, a ROC curve was constructed for each sampling point. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated at different selected cut-off values for each marker and for combinations of markers.

Results:-

In this study, 40 neonates with sepsis (Sepsis Group, 20 confirmed and 20 clinically suspected) and 20 healthy neonates (Control Group) were investigated. Characteristics of the participants are shown in Table 1 and Table 2. There were no significant differences in gender and between the two groups (chi square test; p>0.05). In addition,

there were no significant differences between the groups with respect to mean gestational age, age and birth weight (Student's t-test; p>0.05). The age of onset of sepsis ranged from day 1 to day 30.

Table (1):- Characteristic of studied groups:

		Confirmed Sepsis group		Clinical Sepsis group		Control Group		X^2	P-value
		N	%	N	%	N	%		
Gender									
	Male	**14**	**70%**	**12**	**8**	**11**	**55%**	**0.987**	➢ **0.05**
	Female	**6**	**30%**	**60%**	**40%**	**9**	**45%**		

Table (2):- Demographic characteristic of studied groups:

	Confirmed Sepsis		Clinical Sepsis group		Control Group		F. test	P-value
	Mean	SD	Mean	SD	Mean	SD		
Gestational age (week)	**36.15**	**2.54**	**35.50**	**2.42**	**37.10**	**1.68**	**2.569**	**> 0.05**
Birth weight (Kg)	**2.65**	**0.82**	**2.58**	**0.64**	**2.51**	**0.61**	**0.202**	**> 0.05**
Age (days)	**7.85**	**4.08**	**6.65**	**4.87**	**6.55**	**4.43**	**0.523**	**> 0.05**

Tweenty patients (confirmed sepsis) had positive blood culture in the neonatal sepsis group. Klebsiella pneumoniae (n=7, 35%), Pseudomonas aeroginosa (n=4, 20%), CONS (n=3, 15%), E. coli (n=2, 10%), Staph.Aureus (n=2, 10%), Enterobacter (n=1, 5%) and Candida albicans (n=1, 5%).

Table (3):- Causative organisms of neonatal sepsis:

Blood Culture	No	%
Gram positive organisms	5	25%
Include:		
Coagulase –ve staph	3	15%
Staph.Aureus	2	10%
Gram Negative organisms	14	70%
Include:		
Klebsiella	7	10%
Pseudomonas	4	35%
E.coli	2	5%
Enterobacter	1	20%
Fungal infection	1	5%
Include:		
Candida albicans	1	5%
Total	**40**	**100**

Polymorphonuclear elastase levels, CRP levels, I/T ratio, white blood cell count and platelet count in the Sepsis and Control Groups are shown in Table 5. We found that mean serum PMN elastase levels, CRP levels and I/T ratio were significantly higher in the Sepsis Group than those in the Control Group (Student's t-test; p<0.001).

Table (4):- Comparison between PML elastase and other septic parameter:

	Confirmed Sepsis group		Clinical Sepsis group		Control Group		F. test	P-value
	Mean	SD	Mean	SD	Mean	SD		
PML Elastase (ng/ml)	**1204**	**791.2**	**1231.5**	**750.05**	**90.65**	**33.65**	**21.365**	**0.001***
CRP (mg/dl)	**60.3**	**32.93**	**49.65**	**34.58**	**8.85**	**10.77**	**18.464**	**0.001***

Figure (1):- Comparison of PML elastase in studied groups:

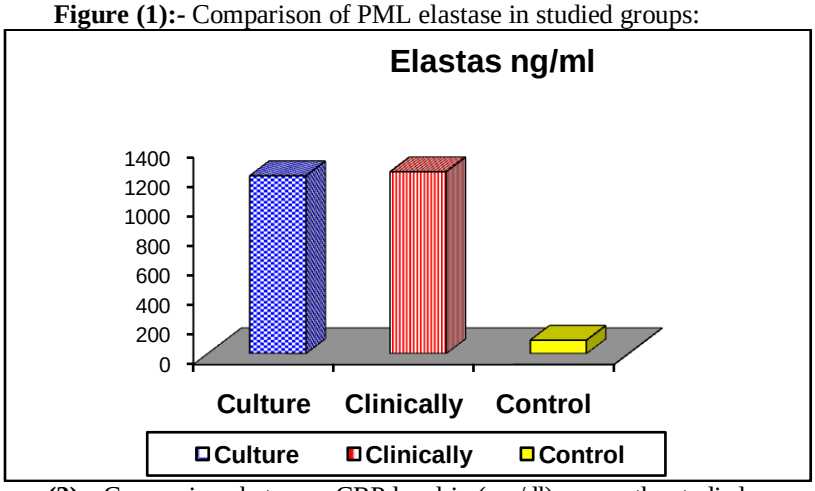

Figure (2):- Comparison between CRP level in (mg/dl) among the studied groups:

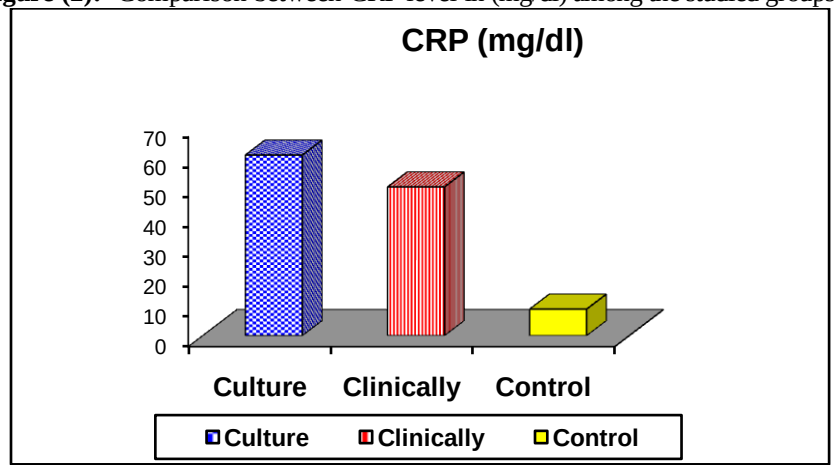

Figure (3):- PML elastase in culture proven group:

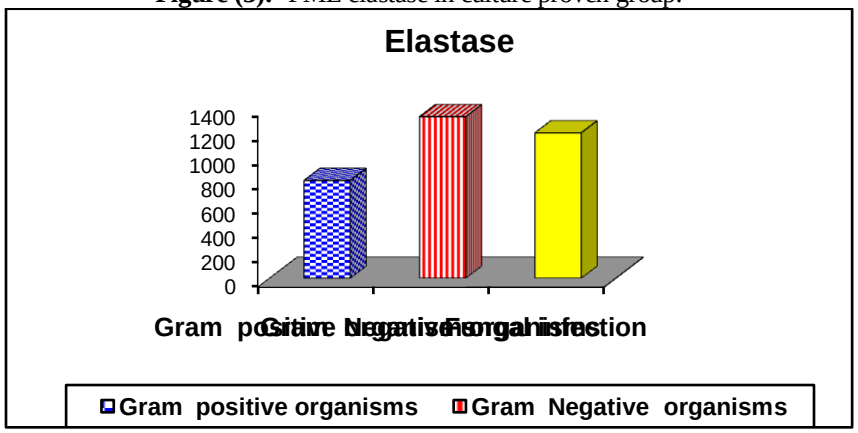

Figure (4):- Hemoglobin level, , total leukocytic count, I/T ratio, platelet count and HSS among the studied groups.

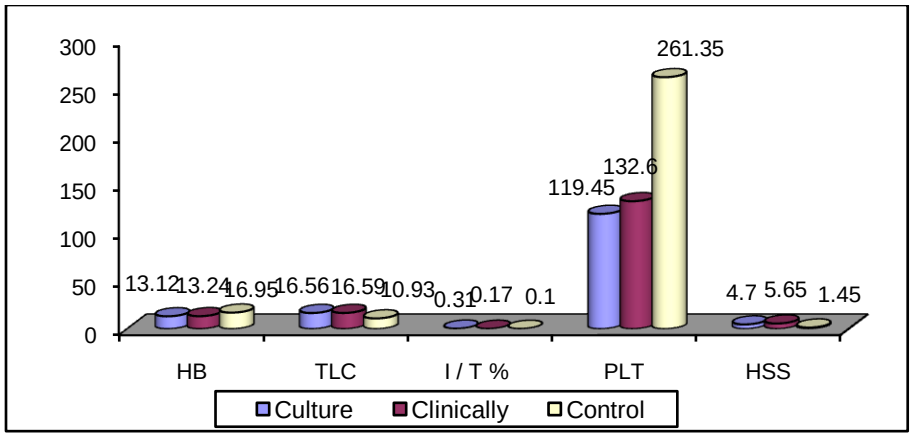

Table (5):- Sensitivity, Specificity, Predictive values and Area Under the Curve of laboratory markers in early diagnosis of sepsis.

Variable	Cutoff	Sens%	Spec%	PPV	NPV	AUC	95%CI	P
PMN Elastase	≥180	96%	92%	94%	90%	0.97	0.94-1.04	<0.001*
CRP	≥6	70%	90%	86%	54%	0.85	0.74-0.95	0.001*
TLC (10^3)	≥20	84%	82%	80%	82%	0.2	0.88-1.0	<0.001*
IT Rtio	≥0.2	86%	84%	80%	82%	0.92	0.78-1.0	<0.001*
Culture	Positive	92%	100%	100%	76%	0.95	0.9-1.0	<0.001*
PLTs (10^3)	≤135	82%	80%	78%	72%	0.94	0.9-1.0	<0.001*
HSS	≥3.5	87%	86%	90%	82%	0.97	0.94-1.0	<0.001*

The specificity, sensitivity, negative predictive value (NPV) and positive predictive value (PPV) were identified using ROC analysis. As shown from Table 5, the highest sensivity (96%), specificity (92%), PPV (94%) and NPV (90%) were found for PMN elastase levels in prediction of sepsis. The sensitivity of CRP in detecting sepsis was 70%, its specificity was 90%, its positive predictive value was 80% and its negative predictive value was 54%. IT ratio had a high specificity and PPV, but lower sensitivity and NPV.

Discussion:-

In this study, 40 septic neonates and 20 healthy neonates were investigated to evaluate the value of serum PMN elastase in determining early diagnosis of neonatal sepsis. It was established that mean serum PMN elastase values before treatment were significantly higher in septic neonates compared with healthy ones, and PMN elastase had high sensitivity and specificity in the early diagnosis of neonatal sepsis. PMN elastase detection was particularly valuable in determining early diagnosis .

Neonatal sepsis is still a leading cause of mortality in neonatal intensive care units all over the world. Early diagnosis and treatment of the newborn infant with suspected sepsis are essential to prevent severe and life threatening complications ***(Stocker et,2010).*** Since the symptoms and findings are nonspecific, neonatal sepsis diagnosis is quite difficult. There is a great need for new diagnostic laboratory methods for the early diagnosis of the disease and the evaluation of prognosis and treatment efficacy.

Clinical manifestations are nonspecific and laboratory parameters such as WBC count or I/T ratio are of limited value in identifying infected newborns **(*Krediet et al,1992*).** Blood culture is the gold standard laboratory technique for the diagnosis of infection, but culture results may take 48-72 hour, and culture positivity rates range from 8% to 73% ***(Mishra et al, 2006).*** False negative culture may also occur ***(Stocker et,2010).*** As a consequence, appropriate diagnosis and therapy could be delayed, worsening the prognosis of the patient **(*Krediet et al,1992*).**
For years, investigators have searched for a test or panel of tests able to identify septic neonates accurately and rapidly while awaiting culture results, in order to obtain an early diagnosis and develop a specific effective treatment for a successful outcome. When high mortality of neonatal sepsis is taken into consideration, it is desirable for the ideal diagnostic tests with maximal sensitivity and negative predictive value. To minimize the unnecessary use of antibiotics in false-positive cases, a diagnostic marker also needs to have reasonably high specificity and a good PPV ***(Chirico and Loda ,2011).***

Hematological parameters have been evaluated in previous studies. WBC count, total neutrophil count, immature neutrophil count, I/T ratios, platelet count are the indices most commonly used. These 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 become positive and the significant influence of non-specific factors. However, I/T ratio of >0.2 may reach a sensitivity of 90% and negative predictive value of 98% ***(Chirico and Loda ,2011).*** In this study, WBC, I/T and platelet count showed low detection sensitivity, specifity, NPV and PPV in neonatal infection ($p>0.05$) than our parameter (PML elastase).

Various published studies have shown PMN elastase to be a useful marker of early infection in the newborn. ***Tsaka et al.*** showed that septic newborns had significantly increased PMN elastase levels at the time of recognition of infection. ***Jensen et al.*** and ***Wojsky et al.*** found that PMN elastase is higher in septic than in nonseptic newborns. In this study, it was detected that PMN elastase levels of newborns with sepsis were significantly higher than controls ($p<0.001$). ***Lawskoska et al.*** also found that in full-term neonates cord blood neutrophil elastase is a good marker of infection.

Wojsky et al reported that the sensitivity and a specifity of serum PMN elastase in the early diagnosis of neonatal sepsis were 76%, 81%, respectively. In our study, the sensitivity of PMN elastase in the diagnosis of neonatal sepsis was 96%, specificity was 92%, PPV was 94% and NPV was 90%. CRP results for sepsis were 70% sensitivity, 90% specifity, 80% PPV and 54% NPV. The results of this study showed that the sensitivity, specifity, PPV and NPV of the PMN elastase were high for early diagnosis of neonatal sepsis. Our findings suggest that PMN elastase is an almost perfect marker and more sensitive and specific than CRP in the diagnosis of neonatal sepsis. However, lack of correction for reference ranges for neonatal PMN elastase values may influence the outcome of PMN elastase as a marker for bacterial infection. In addition, methodological difficulties in detecting PMN elastase and the absence of their routine usage in all centers have limited its use in daily practice.

Conclusions:-

Early diagnosis and management of neonatal sepsis are important for better outcome.
Polymorphonuclear Elastase enzyme being raised early in sera of infants with neonatal sepsis, should be used as an early diagnostic parameter in neonates with suspicion of sepsis especially in high risk group, which could allow earlier initiation of antibiotic therapy with corresponding improvement in outcome.

Recommendations:-

1- Further studies on Polymorphonuclear Elastase enzyme on a larger scale are recommended to verify its diagnostic value in neonatal sepsis. Also the inclusion of neonates of variable gestational ages to search for a possible effect of fetal maturity on Polymorphonuclear Elastase enzyme.
2- Polymorphonuclear Elastase enzyme should be studied in other pathological conditions of the neonate such as trauma and asphyxia to check the specificity of elastase in neonates.
3- A large, well designed prospective multi-center study to confirm the diagnostic role of serum PMN elastase in neonatal sepsis.
4- Further research to discover more sensitive and cheaper tools to identify neonates with sepsis.

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