Evaluation of Some Immunological Tests for Early Diagnosis of Bacterial Neonatal Sepsis

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الخلاصة

يعتبر الأنتان الدموي من أهم الأسباب التي تؤدي إلى الوفاة عند حديثي الولادة بالرغم من الإجراءات الحديثة في وحدة العناية المركزة أجريت دراسة مقطعية (cross-suction study) على 69 من حديثي الولادة الذين تظهر عليهم علامات الأنتان الدموي من بين الراقدين في وحدة العناية المركزة في مستشفى الأطفال والنسائية التعليمي بمدينة الديوانية، و 20 طفل من حديثي الولادة من الأصحاء كمجموعة تحكم ولفترة من آذار حتى تشرين الأول 2009، أجريت هذه الدراسة لتقييم القيمة التشخيصية لبعض العلامات المناعية مثل (CRP, α, IL-6, IL-8, TNF-α) في التشخيص المبكر عن الأنتان الدموي عند حديثي الولادة. وجد دليل المزرعة الايجابية في 20 (28.9%) من حديثي الولادة المشكوك بهم الأنتان الدموي، والذكور أكثر تأثرا من الإناث بين المصابين بالأنتان الدموي وجد أيضا أن متوسط تركيز (CRP, TNF-α, IL8, IL6) عند الأطفال المصابين بتسمم الدم ألسريري المؤكد عند مقارنتهم بالأصحاء، ووجد أن (TNF- α ≥ 65.4 pg/l) (α، و يليه IL-8 60.9pg/l) هم (IL-6، CRP، مع حساسية (80%) و (75%) على التوالي القيمة التنبؤية السلبية (NPV) (97 %) و (94.1%) على التوالي عندما يستخدمان كاختبارات للتنبؤ عن حالات الأنتان الدموي المؤكدة والتفريق بينها وبين حالات الإنتان المشكوك به.

Abstract

Neonatal sepsis is a major cause of death in newborns despite sophisticated neonatal intensive care. This cross-section study was done on 69 neonates with suspected sepsis who were admitted in Neonatal Intensive Care Unit (NICU) of Maternity and Child Teaching Hospital at AL-Diwaniya city, and 20 healthy neonates as a (control group) in the period from March to October 2009.

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This study was conducted to evaluate the diagnostic value of some immunological marker such as (CRP,IL-6, IL-8,TNF-α, in the early diagnosis of neonatal sepsis prior to the blood culture (the golden standard test). The culture positivity rate 20 (28.9%) among suspected sepsis neonates, the male more affected than female among proven sepsis. it was found that the median concentration of CRP, TNF-α, IL-8, IL-6 decreased in order of definitive infection, clinical sepsis and healthy subjects respectively (P<0.001), and found that the TNF-α (≥65.4pg/l) followed by IL-8 (≥60.9pg/l) were better than IL-6 and CRP with sensitivity of (80%) and (75%) respectively and negative predictive value of (97%) and (94.1%) respectively when used as a tests to predict culture positive sepsis cases differentiating them from culture negative sepsis cases .

Introduction

Blood infections remain an important cause of mortality and morbidity in hospital neonatal units.The early signs of sepsis in the newborn are non specific.The frequency of neonatal bacterial infections varies in different countries .Some factors as preterm with low birth weight increase mortality ranging from 10 up to 50% (1).Clinical diagnosis of sepsis is troublesome. The results of blood culture are not available before 48-72 h and usually the antimicrobial therapy has to start before the laboratory results become available. A common approach is to start microbial therapy to all infants with clinical or laboratory signs of blood infection as well as to infants with high risk of early onset sepsis. This may lead to unnecessary increased antibiotic consumption, a higher incidence of side-effects due to their use, increased resistance to antibiotics,a long hospitalization,and separation of infants from their mothers and increased health costs (2).Sepsis and endotoxin activate monocytes, macrophages, lymphocytes, fibroblasts, and endothelial cells to produce and secrete interlukine-1(IL-1),tumor necrosis factor-α (TNF-α), α-

interferon, interlukine-6 (IL-6), interlukine-8 (IL-8), and other proinflammatory cytokines.IL-6,stimulated by TNF-α, IL-1, and endotoxin of bacterial infections, acts as a T-cell activation indicator, induces antibody secretion by human B-cells, causes differentiation of cytotoxic T-cells, and also has the ability to inhibit TNF-α production. Moreover, IL-6 is the major stimulant in hepatic protein synthesis, that is, CRP and fibrinogen during acute phase responses(3).Alternatively, researchers started to look into possible rapid competent diagnostic markers of neonatal sepsis to make a clear distinction between neonates who have clinical signs because of serious bacterial sepsis and others who also have almost similar signs but of other non-infectious etiologies. Examples of these markers, include several interleukins, TNF, procalcitonin (PCT), immunoglobulins and others (4). The objective of this study is to evaluate some immunological tests for early diagnosis of bacterial neonatal sepsis.

Patients and Methods

This cross –section study performed on 69 neonates who were admitted to Maternity and Childhood Teaching Hospital at neonatal intensive care unit (NICU) at AL-Diwaniya city, and 20 healthy neonates as a (control group) in the period from March to October 2009.They were evaluated for neonatal sepsis with blood culture and estimation the concentration of C-reactive protein (CRP),Interlukine-6 (IL-6),Interlukin-8(IL-8)and tumor necrosis factor-α(TNF-α).The neonates were categorized on the basis of their clinical presentation, presence of risk factors and blood culture in: 1) Control group and 2) Cases divided into subgroups of proven sepsis and clinical sepsis Table (1).Inclusion criteria were positive clinical signs of sepsis and positive blood culture as a proven sepsis (n=20).Exclusion criteria were congenital malformations, congenital infections associated with the TORCH complex, and refusal of parents. The symptoms were recorded by the resident of pediatrics or neonatologist in the neonatal or

emergency unit. The patients in subgroup clinical sepsis had clinical and laboratory evidence of infection WBC>15000/cmm and/or absolute neutrophil count (ANC)<1000/cmm, but negative blood cultures (n=49). Also twenty healthy infants were recruited as a control group (non-infected newborn was defined as having no clinical or laboratory signs of sepsis. Blood culture, WBC and ANC were determined at the request of the clinicians at initial evaluation. From each neonate with suspected infection, blood sample of 3 to 4cc was taken by venipuncture for blood culture, CRP,IL6, IL-8 and TNF-determinations and other laboratory tests. Plasma was separated by centrifugation and then stored in aliquots at -20˚C until analysis.IL6, IL-8,and TNF enzyme-linked immunosorbent assay kit (USBiological, Human (TNF-α) BioAssay ELISA Kit, United states Biological) was used to determine the serum level of IL6 IL-8 and TNF-α. Plasma CRP concentrations were measured by CRP- Latex slide agglutination (Spinreact, S.A.)Kit.

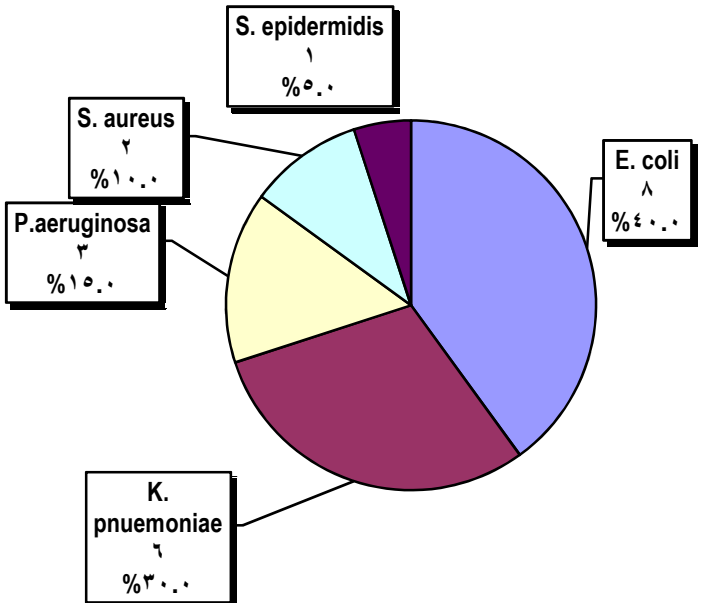
Blood was obtained from two different sit of selected neonates with suspected septicemia by venipuncture under sterile condition, each 0.5ml. of blood inoculated immediately into 20 ml. of brain –heart infusion (BHI) broth contain 0.05% sodium polyanethol sulphonate and inoculated for aerobic and anaerobic conditions**.**

Data analysis: Descriptive and analyzing tests (Mann-Whitney rank-sum test, Student t-test, chi-square test, Pearson correlation and Spearman rank correlation) were performed by using SPSS software. Statistical comparison between the groups (definitive infection group, clinical sepsis group and control group) was performed with analysis of variance (ANOVA) test for Continuous data. Sensitivity and specificity, and 95% confidence interval (CI) values were calculated for TNF-,IL6, IL-8 and CRP. Receiver-operating characteristic (ROC) curves were used for the determination of thresholds for the sepsis group versus healthy neonate group and for the sepsis group versus clinical sepsis group.

The correlation coefficient and its 95% CI are presented. A *P*-value of <0.05 was considered statistically significant.

Results

A total of 89 neonates included in, out of which 69 neonates were with suspected sepsis and 20 healthy neonates (control group).Among suspected sepsis neonates, 20(28.9%) neonates were with positive blood culture (proven sepsis group) and 49(71.1%) neonates were with negative blood culture (probable sepsis group).The blood culture profile among proven sepsis, twenty were blood culture positive: sixteen for gram negative bacilli, eight for Escherichia coli, six for *Klebsiella pneumoniae*, three for *P.aeruginosa*, two for *S. aureu* and one for *Staphylococcus epidermidis*. Figure (1).In this study and among proven sepsis group, the males tend to be more affected (60%) when compared to the females (40%), with 3:2 ratio.But this is not significant with respect to the culture positivity,Table (1).The median concentrations of CRP was statistically different among the three studied groups ,and it has a sensitivity of (70%) and NPV (94.0%) at cut-off 6 mg/l, when compared positive and negative culture sepsis neonates. The median concentration of TNF-***α***, IL-6, and IL-8, were elevated in septic than in non septic newborns, and there was significant difference between them ($p<0.001$) Table(2).Table (3) revealed that there was a sensitivity of 80%, specificity of 70.8%, positive predictive value (PPV) of 96.i%, and negative predictive value (NPV) of 97% at a cut-off value of 65.4 pg/ml for the TNF- assay at presence of signs and symptoms. Also IL-8 concentration had sensitivity of 75%, Specificity of 52.1%,NPP of 94%, and PPV of 94.1% (Fig. 2)**.** The higher ROC area with statistically significant for the TNF (0.877) followed by (0.730) and (0.706) for IL8 and CRP respectively ,with lower ROC area and no statistically significant for IL6 (0.633) when they used as a test to predict culture positive sepsis cases differentiating them from culture negative sepsis cases, Figure (2).



Figure(1):Pie Chart Showing the Relative Frequency of Selected types of Bacterial Isolates Among Culture Positive Neonatal Sepsis cases.

Table (1): The Difference in Gender Distribution by Study Group.

	Proven sepsis (Culture positive)		Probable sepsis (culture negative)		Healthy controls		P (Chi-square)
Gender	NO.	%	NO.	%	NO.	%	
Female	8	40	17	34.7	6	30	0.8[NS]
Male	12	60	32	65.3	14	70	
Total	20	100	49	100	20	100	

NS=Non signifigant.

Table(2):The Difference in Median concentration of Selected Serologic Parameters between the 3 study groups.

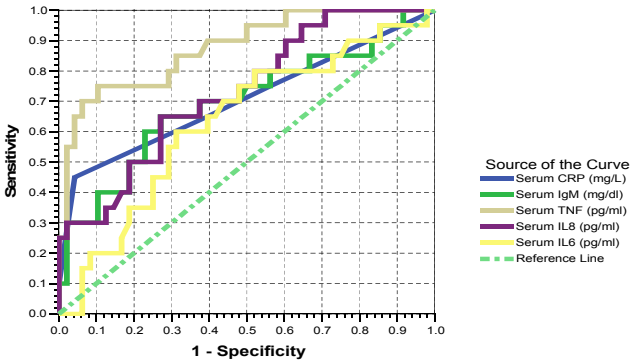
	Culture positive neonatal sepsis	Probable sepsis (culture negative)	Healthy controls
Serum CRP (mg/L) Range Median Inter-quartile range N	(12 to 196) 48 (15 to 96) 20	(12 to 96) 24 (12 to 48) 49	(0 to 96) 12 (3 to 24) 20
P (Kruskall-Wallis) for difference between 3 groups=0.001 P (Mann-Whitney) for difference in median between: Culture positive x Culture negative cases=0.008 Culture positive x Healthy controls=0.001			
Serum TNF (pg/ml)			
Range Median Inter-quartile range N	(108.1 to 1085.6) 321.8 (219.4 to 494.5) 20	(13.3 to 482.9) 146 (82 to 213.9) 48	(0 to 22.7) 0.4 (0 to 9.1) 20
P (Kruskall-Wallis) for difference between 3 groups<0.001 P (Mann-Whitney) for difference in median between: Culture positive x Culture negative cases<0.001 Culture positive x Healthy controls<0.001			
Serum IL8 (pg/ml)			
Range Median Inter-quartile range N	(316 to 1677.9) 685.7 (475 to 968.6) 20	(0 to 969.3) 481.8 (273 to 685.7) 49	(0 to 37.9) 0 (0 to 12) 20
P (Kruskall-Wallis) for difference between 3 groups<0.001 P (Mann-Whitney) for difference in median between: Culture positive x Culture negative cases=0.004 Culture positive x Healthy controls<0.001			
Serum IL6 (pg/ml)			
Range Median Inter-quartile range N	(12.4 to 362.4) 176.2 (102.1 to 286.8) 20	(0 to 712.4) 102 (65.1 to 239.8) 49	(0 to 7.1) 0 (0 to 0) 20
P (Kruskall-Wallis) for difference between 3 groups<0.001 P (Mann-Whitney) for difference in median between: Culture positive x Culture negative cases=0.07[NS] Culture positive x Healthy controls<0.001			

Table(3):Validity Parameters for Selected Serological Parameters when Used as a Test to Predict Culture Positive Sepsis Cases Differentiating them from Culture Negative Sepsis Cases.

Positive if ≥ cut-off value	Sensitivity	Specificity	Accuracy	PPV ate pretest probability =		NPV ate pretest probability = 10%
				50%	90%	
Serum CRP (mg/L) 6	70.6	43.7	52.9	57.1	92.3	94.0
Serum TNF (pg/ml) 65.4	80.0	70.8	73.5	73.3	96.1	97.0
Serum IL8(pg/ml) 60.9	75.0	52.1	58.8	61.0	94.0	94.1
Serum IL6 (pg/ml) 22.1	60.0	60.4	60.3	60.6	93.2	93.1

Table (4): ROC Analysis for Selected Serological Parameters when used as a Test to Predict Culture Positive Sepsis Cases Differentiating them from Culture Negative Sepsis Cases.

serological parameters	ROC area	P
Serum TNF (pg/ml)	0.877	<0.001
Serum IL8 (pg/ml)	0.730	0.003
Serum CRP (mg/L)	0.706	0.008
Serum IL6 (pg/ml)	0.633	0.09[NS]



Figure(2):ROC Curve Showing the Trade-off Between Sensitivity and 1-specificity for Selected Serological Parameters when used as a Test to Predict Culture Positive Sepsis Cases Differentiating them from Culture Negative Sepsis Cases.

Discussion

The blood cultures are positive in only 5%–10% of suspected sepsis cases, even at highly resourced facilities (5). In present study, the rate of proven sepsis was (28.9%) which is lower than that observed by other in which the isolating rate was (40.3%) (6),but comparable with studies which conducted that the positivity rate were (21.5%). The most commonly isolated organisms from the blood of neonates were Gram-negative organisms accounting (85%) of cases predominantly *E. coli* (40%), *K.* pnuemoniae (30%), *P.aeruginosa* (15%). The isolated Gram-positive organisms accounted for (15%) only, mainly *S. aureus*. (10%) , *S. epidermidis* (5%) Figure (1). Increased prevalence of Gram- negative sepsis as found in our study could be due to invasive medical procedures (e.g. endotracheal intubation, umbilical catheterization), indiscriminate and inappropriate use of antibiotics, poor sanitation conditions and ineffective infection control in the maternity services (7).

In the past few decades, it has been observed that several mediators of inflammation tend to become elevated during sepsis.The concentrations of some pro-inflammatory cytokines, especially TNF-α,IL-6, and IL-8, in systemic circulation were reported to increase in severe infections and septic shock (8). Another characteristic of the markers is that they are used in the diagnosis of neonatal sepsis as it gives information about the prognosis of the disease and helps in coming to a decision as to whether to stop or continue antibiotic treatment (9) so these cytokines are promising diagnostic markers and their levels are increased early in the infective process (10).The present study showed that the median concentration of CRP,TNF-*α*,IL-6,and IL-8, were elevated in septic than in non septic newborns, there was significant difference between them ($p<0.001$) Table (2).Present result similar to that which concluded that the level of some pro-inflammatory cytokines were higher in neonates with bacterial infections (11).The median concentrations of CRP was statistically different among the three studied groups, single CRP value has a sensitivity of (70%) and NPV (94.0%) at cut-off 6 mg/l, when compared positive and negative culture sepsis neonates, which is comparable to another study which revealed that the sensitivity was (60%) , and the NPV was (95.7%) (12).The study of TNF-α, cytokine that is synthesized at the beginning of the inflammatory cascade has rendered differing results. In present study, the serum TNF-*α* level were significantly increased in newborns with sepsis, for the cut-off value of (65pg/ml), with sensitivity, specificity, accuracy, PPV and NPV were (80%), (70.8%),(73.5%), (96.1%) and (97%) respectively, when compared the culture positive with culture negative sepsis neonates. In a study conducted on 26 neonates with blood culture positivity and clinical sepsis, and 29 healthy neonates, found that the sensitivity, specificity, accuracy, PPV and NPV were (100%), (96.6%),(98.3%), (96.2%) and (96.5%) respectively with conclusion that the

TNF-α is the best marker in the diagnosis of neonatal sepsis and this marker is also valuable in following the effectiveness of treatment and determining the prognosis of the disease (13). Interleukin-8 is a cytokine that has a role in the release, activation, and chemotaxis of neutrophils.Serum IL-8 level has been reported to increase in neonatal sepsis and have a sensitivity of about (70–90%) and a specificity of about (50–95%) (14).It was detected that IL-8 concentration higher in both infected and non- infected infants with signs of infections than in healthy (controls) neonates with statistical significantly difference between infected, non-infected and healthy neoborns ($P < 0.001$), and provided some diagnostic accuracy, with a sensitivity of (75%), specificity of (52.1%), PPV of (94%), and NPV of (94.1%) at a cut-off value of (60.9 pg/ml) for the IL-8 assay .Interleukin-6 has been reported as an early indicator of neonatal sepsis because of its rapid increase after endotoxin challenge and secreted by monocytes and macrophages in response to bacteremia (15). In present study, it was observed that IL-6 concentration in newborns with culture-proven sepsis and culture-negative sepsis were higher than healthy(controls) newborns, but with no statistical significantly difference between culture positive and culture negative sepsis neonates (P 0.07), with a sensitivity of (60%), specificity of (60.4%),PPV of (93.2%), and NPV of (93.1%) at a cut-off value of (22.1 pg/ml) . Present study comparable with previous study which conducted on 35 infants with culture proven sepsis and 37 infants with clinical sepsis and reported that the predictive value for IL6 assay ranging from (50%-82%) for Sensitivity, (56%-90%)for Specificity, (60%-91%) for PPV, and (46%-80%) for NPV (16) . Others found that the Chemo-attractant IL-8 with a sensitivity of (92%) and specificity of (70%), NPV of (94%) appears to be a better marker of neonatal sepsis than IL-6 (17) The discrepancy between the studies may be partly related to the different detection limits of the assay kits.

Receiver operating characteristics (ROC Analysis) for the serological tests: To test the validity of the serological markers in the presence of a probable sepsis group, ROC curve was done to compare sepsis group with probable sepsis group, Table (3) Figure(2). The area under the curve (AUC) was used as an estimation of the overall diagnostic accuracy .It was found that the TNF-α, followed by IL8 were AUC (0.877) and (0.73) and (P value were <0.001 and 0.003) respectively, were superior to CRP, and IL6 in distinguishing between neonates with sepsis and probable sepsis. Another study found that the AUC for TNF-α,CRP,IL6 and IL8 were (1.00),(1.00),(0.97),(0.90) and (0.68) respectively and conclude that the TNF-α is a superior marker in the diagnosis of neonatal sepsis (13). IL8 superiority to CRP in the detection of neonatal sepsis and concluded that the Serum IL8 level was significantly higher in infants with confirmed infection than healthy newborns (18).

Conclusion

Serum levels of TNF-α and IL-8 which are a mediator of inflammation with high sensitivity and specificity, and can be used to diagnosis the neonatal sepsis.

Recommendations

Introduce modern technique such as ELISA in our hospitals for early diagnosis of neonatal sepsis, by cytokines profiles such as IL-8 and TNF-α.

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