EARLY PREDICTION OF GRAM-POSITIVE BACTEREMIA IN FEVERISH EGYPTIAN PATIENTS USING SOME SELECTED INFLAMMATORY MEDIATORS

BY

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ABSTRACT

Bloodstream infection is an important cause of morbidity and mortality. Some components of the bacterial cell wall bind to receptors on the cell surface of the host monocytes and macrophages and induce cytokines production. This study assessed the levels of IL- 6 and TNF-α in 36 feverish Egyptian patients, with Gram positive bacterial infections. For early prediction of the infection, blood culture samples from feverish patients were collected from different medical hospitals in Cairo, Egypt. The recovered Gram-positive isolates were identified as *Staphylococcus* species and *Micrococcus* species. For patients, IL-6 and TNF-α were measured in the patients' sera by ELISA. Antimicrobial susceptibility was determined for the tested isolates using 16 antibiotics by Kirby Bauer disk diffusion. Cell membrane protein profile was performed to detect the bands responsible for TNF- α production. The IL-6 and TNF-α in sera of all patients, with Gram-positive bacterial infections, showed significantly ($P < 0.05$) higher levels as compared to that of their counterparts with negative bacterial blood cultures. Tested isolates showed antimicrobial resistance to most tested antibiotics. The bacterial cell membrane proteins bands between 29-36 kDa were detected in the tested isolates. Some Inflammatory cytokines (IL-6 and TNF-α) can be used as valuable tools for early prediction of Gram-positive bacterial infections even before culture results are available.

Key words: Bacteremia; fever; Il-6; Staphylococci; TNF-α.

Introduction

Interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF- α) are two cytokines produced by activated macrophages in response to injury. They mediate several systemic changes associated with trauma or infection such as fever, neutrophilia, and increased hepatic acute phase protein synthesis (Lukaszewski *et al.*, 2008). There had been a shift from the predominance of Gram-negative bacteria to predominance of Gram-positive bacteria over the past two decades (Horasan *et al*., 2011), with an increasing proportion of multi-resistant bacteria and higher recovery of coagulase

negative staphylococci (CoNS) (Rosa *et al*., 2014). CoNS are one of the most common infectious agents responsible for nosocomial blood stream infections, especially when patient is on indwelling devices and premature neonates with low birth weight. Therefore, CoNS should not be considered as contaminants as their pathogenic role in nosocomial blood stream infection is continuously proven in different clinical situations (Parashar, 2014). Although *Micrococcus* is generally thought to be a saprotrophic or commensal organism, it can be an opportunistic pathogen, particularly in hosts with compromised immune systems (Kocur *et al*., 2006). *Micrococcus spp.* has been frequently isolated from blood cultures and could represent a cause of infections associated with medical devices (Martin *et al.*, 2008; and Ramos *et al*., 2009). Peptidoglycan (PepG) and lipoteichoic acid (LTA), two of the major cell wall components in Gram-positive bacteria, have been shown to stimulate the release of some inflammatory mediators (Wang *et al*., 2000), also the membrane of *S. aureus* L form was capable to stimulate TNF-α production by macrophages (Kuwano *et al*., 1997). The increasing worldwide prevalence of antibiotic-resistant staphylococcal strains renders their infections very difficult to treat (Giai *et al*., 2013). The worldwide mortality associated with *S. aureus* bacteremia ranges from 30% to 50% (Kotsaki and Giamarellos-Bourboulis, 2012).

Since the majority of feverish patients are treated empirically with broad-spectrum antibiotics as soon as the fever develops, the use of inflammatory markers for early detection of bacterial infections might be essential to reflect the nature of the infection and to guide the choice of more specific antibiotic therapies, even before the culture results are available (Tavares *et al.*, 2005). The present study was established to assess the levels of IL-6 and TNF-α in feverish patients for early diagnosis of Gram-positive bacterial infections and for prevention of complications associated with such infections.

Materials and Methods

Blood Sampling

Thirty six blood samples were collected by authoritative medical staff of the clinical laboratories (Al-Abasia Fever Hospital, Cairo, Egypt) on fever onset from outpatients according to the standard protocols of these medical institutions. This study was approved by local ethics committees. Collected blood samples were subjected to blood cultures and total white blood cells (WBCs) counting immediately after samples collection. Blood samples were also centrifuged and the separated sera were stored at -20°C until use for assays of cytokines and CRP level.

Microbial cultures

Two blood samples (5 ml each, were collected separately during the same episode of fever from each patient with sterile precaution) were immediately injected into two ready-made purchased blood culture bottles, mixed, and incubated at 37^{o}C. The bottles showing no turbidity after 14 days of incubation were discarded, while those showing turbidity within the 14 days were subjected to subculture onto nutrient agar. The inoculated plates were incubated at 37^{o}C for 24 h as described previously (Komar,

2013). Identification of isolates was carried out at National Cancer Institute (Cairo, Egypt) using, automated system for identification to species level, MicroScan Walk Away 96 SI System (Dade Behring, Germany) and API Staph System (Biomerieux, Marcy-L'etoile, France) according to the instructions of the manufacturers.

Biological assays

Total WBCs

Total WBCs were determined for plasma samples using Micros 60 counter (Horiba Diagnostics, USA) after blood collection in EDTA-containing tubes (Tavares *et al*., 2005).

Serum CRP

CRP was determined using AVITEX® CRP (Omega Diagnostics, Hillfoots, UK) as described previously (Fisher and Nakamura, 1976).

Human IL-6 and TNF-α levels

Levels of IL-6 were measured in the sera collected from the patients and stored at -20^{o}C, after blood sample centrifugation, as well as healthy volunteers under the same conditions by using an enzyme linked immunosorbent assay kit Human IL-6 ELISA KIT (RayBio Inc, Georgia, USA) (Van Oers, 1993). Levels of TNF-α were also measured using Human TNF-α ELISA Kit (AviBion Co, Vantaa, Finland) (Seriolo *et al*., 2006). Instructions of the manufacturers were strictly followed in both assays.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of all isolates to Levofloxacin (LEV, 5µg), Erythromycin (ERT, 15µg), Oxacillin (OXA, 1µg), Chloramphenicol (CLM, 30µg), Rifampin (RFM, 5µg), Amoxycillin-clavulanic acid 2:1 (AMC, 30µg), Vancomycin (VAN, 30µg), Tetracycline (TET, 30 µg), Cephalothin (CPL, 30µg), Azithromycin (AZM, 15µg), Clindamycin (CLN, 2µg), Ofloxacin (OFX, 5µg), Gentamicin (GNT, 10µg), Sulphamethoxazole-trimethoprim (SXT, 25µg), Ciprofloxacin (CIP, 5µg), and Cefotaxime (CTX, 30 µg) was performed using Kirby Bauer disk diffusion method (Hombach *et al.*, 2013). Antibiotic discs were obtained from Oxoid, LTD (Dublin, Ireland). The results were interpreted according to the guidelines of clinical and laboratory standards institute (CLSI) (CLSI, 2010).

Bacterial cell membrane-protein profiles

Membrane proteins of the tested isolates were prepared as reported previously (Stewart *et al*., 2005). Concentration of the membrane proteins were measured as previously described (Upreti *et al*., 2012). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of the membrane proteins was performed as mentioned (Moore et al., 2014). Molecular weights of the separated proteins were determined through using standard wide range (10-250 kDa) prestained protein marker

(Fermentas; Cheshire, UK). Analysis of gel lanes was carried out using AlphaImager™ 2200 software (San Jose, CA, USA).

Statistical analysis

Statistical significance of the obtained data for cytokines levels was determined using *Student's* t-test and P- value *<0.05* was considered significant. Receiver operating characteristic (ROC) curves, plotting sensitivity versus specificity, to evaluate the diagnostic performance of the tested inflammatory mediators. An area under the curve (AUC) closer to 1 indicates greater diagnostic power (Groeneveld *et al*., 2001). Sensitivity, specificity, and positive predictive and negative predictive values for the cutoff points that represented the best discrimination, as derived from ROC curves, were also calculated.

RESULTS

Clinical characteristics

A total of 36 outpatients (20 males and 16 females with age range 20-55 years) were included, 27 positive cases for microbial infection, nine negative cases. Four healthy laboratory personnel were included in this study. Table (1) shows the number and frequency of positive and negative cases for microbial growth from blood culture samples of the patients. Table (2) shows different bacterial species recovered from the patients.

Table 1. Number and frequency of positive and negative cases for microbial growth from blood culture samples of patients

Total no. of	No. of positive blood cultures-%	No. of negative blood cultures-%
36	27-75%	9- 25%

Table 2. Identified Gram-positive bacterial species recovered from the patients.

Bacterial isolate	No. of isolates (n=27)	
S. aureus	12	44.4%
S. epidermidis	5	18.5%
S. hominis	1	3.7%
S. sciuri	7	26%
S. lugdunensis	1	3.7%
Micrococcus spp.	1	3.7%
Total	**27**	

Abbreviations: *S.* = *Staphylococcus*

WBCs count, IL-6, TNF-α and CRP of the tested patients

The total WBCs counts of the patients involved in this study had an average value of $15x10^3/mm^3$ for bacteremic patients relative to $9x10^3/mm^3$ for non-bacteremic patients and healthy control.

Statistical analysis of the data for IL-6 levels showed P- value of 0.0247 in case of bacteremic relative to non-bacteremic patients. For TNF-α levels P-values were 0.0023 for bacteremic relative to non-bacteremic patients. Average levels of IL-6 and TNF-α in patients with bacteremic and non-bacteremic fever, and healthy control (Fig. 1 & 2), respectively.

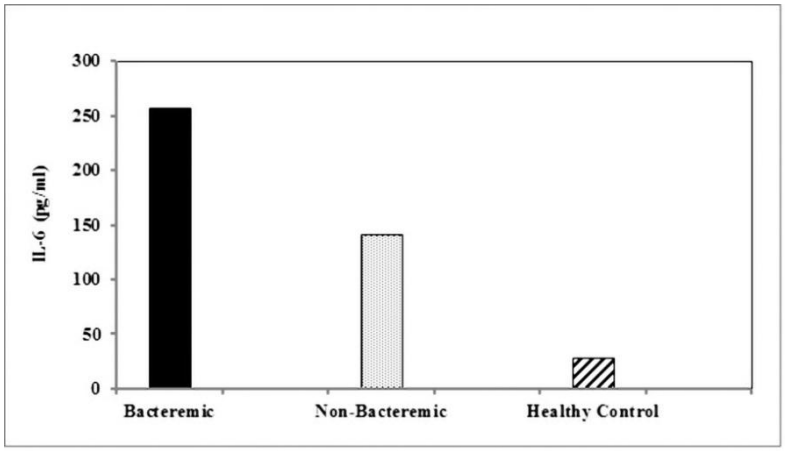

Fig. 1. Average IL-6 levels in patients with bacteremic and non-bacteremic feverish patients as well as healthy control.

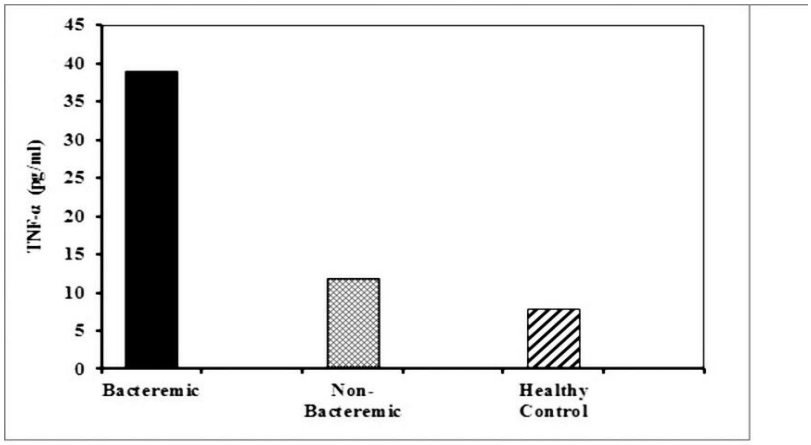

Fig. 2. Average TNF-α levels in patients with bacteremic and non-bacteremic feverish patients as well as healthy control.

The sensitivity, specificity, positive predictive value, and negative predictive value at the best cutoff values of IL-6, and TNF-α based on ROC analysis were

calculated to be (77.8%, 77.8%, 0.77, 0.77, at 157 pg /ml for IL-6 and 100%, 100%, 1, 1, at 13 pg/ml for TNF-α, respectively). The diagnostic properties of IL-6, and TNF-α levels were compared by calculating the areas under the ROC curves (AUC). The AUC of the ROC curve for IL-6 was 0.78 and that for TNF-α was 1.0 ($P < 0.05$) (Fig. 3). This indicates that both IL-6 and TNF-α would be useful markers for prediction of positive blood cultures in feverish patients.

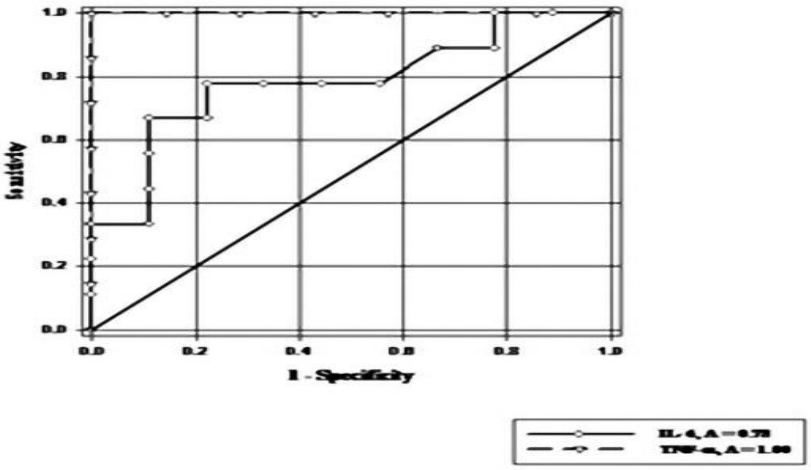

Fig. 3. Receiver operating characteristic (ROC) curves of IL-6 and TNF-α serum levels.

On the other hand, CRP showed strong agglutination in all patients suffering from Gram-positive bacterial infections compared to non-infected patients as well as healthy controls (no agglutination).

Antimicrobial susceptibility

Antimicrobial susceptibility patterns of the Gram-positive isolates to the tested antibiotics showed high resistance levels to ERT, OXA, CLN , and SXT (67%) followed by VAN, and AZM (44%), RFM, AMC, and CPL (33%). On the other hand, lower levels of resistance (< 22%) were observed with LEV, CLM, TET, OFX, and CIP. Furthermore, all the tested isolates were sensitive to GNT and CTX (Fig. 4.)

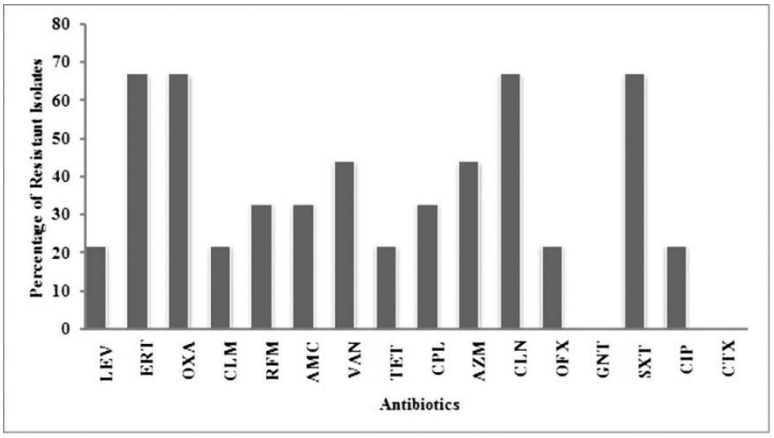

Fig. 4. Resistance of the tested isolates to different antibiotics. Abbreviations: LEV = levofloxacin, ERT = erythromycin, OXA = oxacillin, CLM = chloramphenicol, RFM = rifampin, AMC = amoxycillin-clavulanic acid, VAN = vancomycin, TET = tetracycline, CPL = cephalothin, AZM = azithromycin, CLN = clindamycin, OFX = ofloxacin, GNT = gentamicin, SXT = sulphamethoxazole-trimethoprim, CIP = ciprofloxacin, CTX = cefotaxime.

Cell membrane protein profiles of the tested bacterial isolates

Cell membrane protein analyses of *S. aureus* and *S. sciuri* (mostly isolated species from clinical samples) were carried out. Analysis of the lanes of both isolates showed the presence of bands, their molecular weights ranged between 28-36 kDa which are responsible for TNF-α production (Fig. 5).

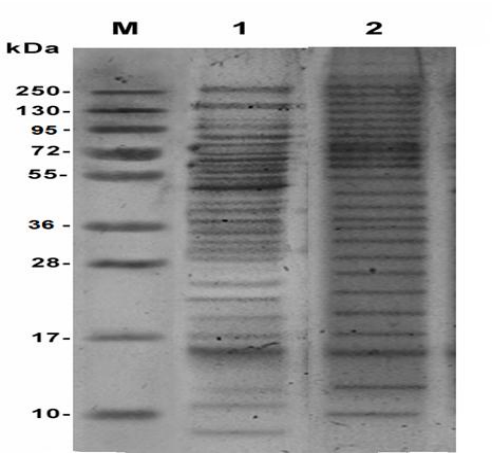

Fig. 5. SDS-PAGE of cell membrane proteins of selected *Staphylococcal species*. Lanes 1 and 2 represent cell membrane proteins of *S. aureus* and cell membrane proteins of *S. sciuri*, respectively. M is molecular weight of standard protein marker.

Discussion

Significant changes in the epidemiology of infections have emerged during the past decade. Gram-positive pathogens have become more predominant. Risk factors such as venous catheters are associated with an increased incidence of Gram-positive infections (Rosa *et al*., 2014).

It has been reported that Gram-positive bacteria induced twice the TNF-α level from human monocytes than Gram-negative bacteria. The principal difference between Gram-positive and Gram-negative bacteria resides in their cell wall structure. Gram-positive bacteria have lipoteichoic acid (LTA) instead of lipopolysaccharide (LPS), and also contain more peptidoglycan in their cell wall as compared to Gram-negative bacteria (Hessle *et al*., 2004). This notion was also supported by a recent study which revealed that stimulation of blood with LPS and LTA for 4 h led to production of IL-6 and TNF-α in adults and neonates, with higher levels in case of LTA (Koch *et al*., 2014). Circulating proinflammatory mediators such as IL-6, and CRP have been suggested to be predictive for a systemic microbial bloodstream infection (Tavares *et al*., 2005). The present study demonstrated a significant increase in serum levels of CRP, IL-6 and TNF-α in patients with positive blood cultures than negative cultures patients. Another study demonstrated that CRP was elevated both in septic group, with relation to the control group, and was also found that TNF-α levels were significantly higher in patients with microbiologically-evidenced infections than in patients with only clinically-evidenced infections (El Wakeel *et al*. 2012).

Escalating bacterial resistance among Gram-positive pathogens means that what were once effective and inexpensive treatments for infections caused by these bacteria are now being seriously questioned. As a whole, multi drug resistant Gram-positive pathogens are rapidly becoming an urgent and sometimes unmanageable clinical problem (Cornaglia, 2009). In the current study, all Gram-positive bacterial isolates, recovered from feverish patienst, showed different resistance levels to the tested antibiotics. Only GNT and CTX represent antibiotics of choice as the tested organisms could not develop resistance against them. Another study also suggested that Cefotaxime showed better susceptibility than other third generation cephalosporin against staphylococci (Jyothsna *et al*., 2011). The sensitivity pattern of *S. aureus* to Gentamicin was 92.4% (Nwankwo and Nasiru, 2011)

It has been reported that cell membrane proteins of 28-36 kDa are responsible for TNF-α induction (Kuwano *et al*., 1997). In the current study, analyses of cell membrane proteins for *S. aureus* and *S. sciuri* revealed the presence of the bands with molecular weights responsible for TNF- α induction in both strains. Further investigation through using Western blotting and implementation of labeled- and band specific-antibodies, are required to confirm this finding. In conclusion, some inflammatory mediators such as IL-6, TNF-α and CRP would be valuable in early prediction of Gram-positive bacterial infections and this may improve the diagnosis and the therapeutic outcomes, even before culture results are available. Further investigations are required to evaluate the potential association between other cytokines and Gram-positive bacterial infections.

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التنبؤ المبكر لتجرثم الدم بالبكتيريا موجبة الجرام في المرضى المصريين المصابون بالحمي عن طريق بعض الوسطاء التهابية المختارة

للسادة الدكاترة

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تعد عدوى مجرى الدم هو أحد أهم أسباب المراضة والوفيات. بعض مكونات جدار الخلية البكتيرية ترتبط بمستقبلات على سطح الخلية من حيدات المضيفة والضامة وتحفز إنتاج السيتوكينات. قيمت هذه الدراسة مستويات IL-6 و α -TNF في ٣٦ من المرضي المصريين المصابون بالحمي مع الالتهابات البكتيرية إيجابية الجرام. للتنبؤ المبكر بالعدوى، تم جمع عينات الدم من المرضى المصابون بالحمي من مختلف المستشفيات الطبية في القاهرة، مصر. صنفت العزلات إيجابية الجرام كأنواع المكورات العنقودية وأنواع ميكروكوكس. بالنسبة للمرضى، تم قياس IL-6 و α-TNF في مصل الدم بواسطة ELISA. تم إجراء إختبار الحساسية للمضادات الحيوية لبعض العزلات المختارة باستخدام ١٦ من المضادات الحيوية عن طريق باور كيربي طريقة نشر القرص. تم إجراء تشخيص بروتين غشاء الخلية للكشف عن العصابات المسؤولة عن إنتاج α –TNF. و أظهرت نتائج IL-6و α- TNF في مصل جميع المرضى، المصابون بالبكتيرية إيجابية الجرام، ($P < 0.05$) مستويات أعلى بالمقارنة مع نظرائهم ذو العينات البكتيرية السالبة. وأظهرت العزلات المختبرة مقاومة لأغلب المضادات الحيوية التي تم اختبارها. تم الكشف العصابات بين ٢٩-٣٦ كيلو دالتون في بروتينات غشاء الخلية البكتيرية في العزلات المختبرة. بعض السيتوكينات الالتهابية (IL-٦ و α-TNF) يمكن استخدامها كأدوات قيمة للتنبؤ المبكر للعدوى البكتيرية إيجابية الجرام حتى قبل ان تكون نتائج مزارع الدم متاحة.