RAPID AND SPECIFIC DIAGNOSIS OF BACTERIAL SEPSIS IN HIGH RISK PATIENTS

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ABSTRACT
Sepsis is a major healthcare problem worldwide, athe accurate and timely detection of sepsis remains a challenge specially in high risk patients as blood culture and sensitivity testing results are slow and delay the beginning of treatment. Discrimination between sepsis and systemic inflammatory response syndrome (SIRS) is still difficult. This study was aiming for rapid diagnosis of sepsis by both polymerase chain reaction (PCR) of 16 S ribosomal DNA gene and to investigate the specific expression of DcR 3 in patients with sepsis by measuring its serum level within the first 48 hours of hospitalization and compare the results of both methods with the traditional blood culture results. This study was performed on 80 subjects (52 subjects suggestive to be bacterial sepsis patients and 28 subjects apparently healthy blood donors as a control). Peripheral venous blood was collected to be utilized for polymerase chain reaction and serum used for measurement of DCR3 by (EIA). Twenty five samples were PCR positive(48%) and 27 samples were PCR negative PCR with sensitivity, specificity, positive predictive value and negative predictive value 100,90, 88 and 100 respectively. The average DCR3 level was 10.35 fold than the control group in sepsis and We recommended that the amplification of 16s ribosomal DNA by PCR providing highly sensitive and time saving diagnostic technique and measurement of DCR3 by EIA provide a noninvasive, specific biomarker for prediction of bacterial sepsis progression and discrimination between bacterial sepsis and systemic inflammatory response syndrome.

KEYWORDS: Sepsis, DcR3, polymerase chain reaction, blood culture.
INTRODUCTION

Sepsis is a major healthcare problem worldwide. It affects millions of people each year and its incidence increases annually. Sepsis has been called a hidden public health disaster. An epidemiologic study reported that septic shock is the most common cause of death in noncoronary intensive care units, and the tenth leading cause of death overall in high income countries.\[^1\]

The accurate and timely detection of sepsis remains a challenge in high risk patients, as differentiation between infectious and noninfectious causes in those patients especially in early stages will help to reduce morbidity and mortality. The identification of causative pathogens through blood cultures is still the gold standard for sepsis diagnosis.\[^2\] However, confirmation of pathogens by cultures is slow, of moderate sensitivity and it often delays the beginning of proper treatment especially for ICU patients. Recently, Molecular techniques such as PCR represent rapid and sensitive methods that overcome disadvantages of blood cultures.\[^3\]

Decoy receptor 3 (DcR3) is a member of the tumor necrosis factor receptor superfamily (TNFRSF), officially designated TNFRSF6b. It was identified in (1998) by the search of genes with homology to the TNFR gene super family in Expressed Sequence Tag (EST) database. DcR3 can bind to the TNF family members FasL, LIGHT(CD258) and tumor necrosis factor like 1 A (TL1A).

It does not bind to other known TNF family members.\[^4\] The role of DcR3 in infection has been studied in depth.\[^5\], had analyzed DcR3 mRNA levels in purified cell populations obtained from peripheral blood mononuclear cell (PBMC) from healthy donors by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). On treatment of these cells with the bacterial antigen lipopolysaccharide (LPS), they respond by significant increase in the DcR3 mRNA level. This observation suggests that the recognition of pathogen associated molecular patterns (PAMP) by antigen presenting cells (APC) might induce DcR3 expression.\[^6,7\]

In 1992, the American College of Chest Physicians (ACCP) / Society of Critical Care Medicine (SCCM) introduced definitions for systemic inflammatory response syndrome (SIRS) as well as sepsis, severe sepsis and septic shock. SIRS can be initiated by ischemia, inflammation, trauma, infection or a combination of several “insults”. SIRS is not always
associated with infection. As trauma, inflammation (anaphylaxis, adrenal insufficiency, low blood volume, heart failure and pulmonary embolism) lead to the activation of the inflammatory cascade by damage associated molecular pattern (DAMP). When SIRS is mediated by an infectious insult, the inflammatory cascade is often initiated by pathogen associated molecular pattern (PAMP).[8]

Aim of the work
This study was aiming for rapid diagnosis of sepsis by both polymerase chain reaction (PCR) of 16 S ribosomal DNA gene and to investigate the specific expression of DcR 3 in patients with sepsis by measuring its serum level within the first 48 hours of hospitalization and compare the results of both methods with the traditional blood culture results.

MATERIALS AND METHODS
Patients and control subjects
The present study was approved by the ethical committee in FMG and carried out from October 2014 to February 2016 in our Microbiology labs on 52 patients and 28 control subjects.

Patients group
52 hospitalized patients admitted with signs and symptoms suggestive of septicemia in intensive care unit in the early 48 hours of admission before receiving antibiotic (31 males and 21 females and their age ranging from 19 to 70 years old) and. Patients: Patients were chosen according to certain inclusion and exclusion criteria. They were collected from El Zahraa (EMOH) and Tanta University Hospitals. As described in the American College of Chest Physicians/Society of Critical Care Medicine (ACCP/SCCM) consensus classification, each one of the patients group had at least two of the four SIRS criteria: (i) body temperature of >38°C or <36 °C, (ii) heart rate (>90 beats per min), (iii) respiratory rate (>20 breaths per min),(iv) total leucocytic counts (TLC) counts >11,000 cells/mm3 or <4,000 cells/mm3. Exclusion Criteria: Patients with HIV,HCV,HBV infection immunocompromised state: uncontrolled diabetes mellitus, active T B, patients receiving immunosuppressive therapy , or those with hematological disorders were excluded from the present study.

Control group
28 Apparently healthy blood donors, negative for all blood screening tests (HIV Ag and Ab, HBsAg, HCV Ab and syphilis), their age and sex matched with that of test group patients.
They are attendance of Tanta Regional Blood Transfusion Center (Egyptian ministry of health(EMOH)).

**Specimens and Methods**

**From the patients group only**

1- From all participants of the study, - 5 mL of peripheral venous blood were drawn aseptically on plain tubes (VOMA MED) within the first 48 hours of admission and serum separation was done by centrifugation at 5000 rpm for five minutes (Hittchi centrifuge). Collected serum was stored at -70 oC in cryotubes till used for measurement of serum level of DcR3 by enzyme immunoassay (EIA). by EIA Kit for quantitative measurement of Human DcR3 (RayBio Human DcR3 EIA Kit USA).

2- 5 mL of peripheral venous blood were drawn aseptically on EDTA tubes (VOMA MED) separated plasma were collected in cryotubes and stored at -20 oC till were used for detection of the bacterial sepsis by amplification of 16S bacterial ribosomal DNA gene by PCR.

**A- DNA extraction from the blood samples**

DNA was extracted from plasma by DNA extraction kit (QIA amp R Blood Mini).

**B- PCR method for amplification of 16S ribosomal DNA gene**

PCR was performed on extracted DNA with the use of Taq Master Mix Kit (QIAGEN cat no 201203). Nuclease free (sterile) water.

Primers of 16S ribosomal DNA (Invitrogen Life Technologies).

One pair of universal primers corresponding to portion of DNA sequence encoding 16S ribosomal DNA 861 bp fragment have been used to define the organism as a bacterium Primer sequence.

Forward. 5´ AGAGTTTGATCCGGCTCAG 3´
Reverse. 5´ GGACTACCAGGGTATCTATT 3´ (9) (10) (11)

**C- Electrophoresis**

The amplification products were identified with electrophoresis on Agrose gel specially purified for gel electrophoresis of nucelic acids (BIOLINE cat no Bio-41026). Electrophoresis buffer (Tris- borate –EDTA) buffer (BIOLINE cat no A0972.1000).
Ethidium bromide (10ug/ml) MP Biomedicals (through Fisher) #802511. Loading dye (Bromophenol Blue, Xylene Cyanol FF).

Thermo Fisher Scientific Molecular weight DNA ladder (HyperLadder 1kb) HyperLadder 1kb produces a pattern of regularly spaced bands, ranging from 200,400,600,800,1000,1500 upto 10,037bp (Bioline United Kingdom).

3-Blood cultures were carried out by staff of the laboratory of the hospital using BACTEC automated blood culture system(3D 60)for at least 3-5 days according to policy and procedures in collection hospitals. The positive bottles were identified by sub culture on blood agar, Mac Conkey agar and biochemical reactions.

CRP, ESR and total leucocytic count (TLC) were carried out as routine investigations in the hospital.

Statistics
Statistical presentation and analysis of the present study was conducted, using the mean, standard deviation and chi-square test by SPSS V.20.

RESULTS
Results of PCR amplification
Amplification of 16S bacterial ribosomal DNA gene by PCR was utilized for the 52 patients plasma. Our results demonstrated that 25/52 were PCR positive (48%) and considered as sepsis group, 27/52 were PCR negative (52%) and considered as SIRS group.

![Agarose gel electrophoresis of product of PCR amplification of 16S bacterial ribosomal DNA gene in sepsis and SIRS.](image)

Fig. (1) Agarose gel electrophoresis of product of PCR amplification of 16S bacterial ribosomal DNA gene in sepsis and SIRS. The picture shows the amplified sequence of
16s rRNA gene 861 bp. The first lane is 1kb ladder (molecular DNA marker) separating 200,400,600,800,1000 1500,2000 bp bands
Lane 2 represents positive control
Lane 3 represents negative control
Lane 4, 6 and 8 represent positive samples
Lane 5 and 7 represent negative sample

So in the present study 3/52 (5.8%) were positive PCR while their blood culture were negative.

Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) results of PCR for detecting sepsis considering blood culture as gold standard were 100% 90% 88% 100% respectively.\[12\]

Table (1): PCR amplification of the 16s rDNA gene from plasma samples in relation to blood culture results.

<table>
<thead>
<tr>
<th>PCR results</th>
<th>Blood culture positive</th>
<th>Blood culture negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>No 22</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>% 100.0%</td>
<td>10.0%</td>
</tr>
<tr>
<td>Negative</td>
<td>No 0</td>
<td>27.0</td>
</tr>
<tr>
<td></td>
<td>% 0.0%</td>
<td>90.0%</td>
</tr>
<tr>
<td>Total</td>
<td>No 22</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>% 100.0%</td>
<td>100.0%</td>
</tr>
<tr>
<td>Chi-square</td>
<td>X² 41.184</td>
<td></td>
</tr>
<tr>
<td>p-value</td>
<td>0.001*</td>
<td></td>
</tr>
</tbody>
</table>

* significant.

Measurement of serum level of DcR3 by sandwich enzyme immunoassay (EIA).

The average DcR3 level was 10.35 and 3.09 folds than the control group in sepsis and SIRS groups respectively.

Serum level of DcR3 increase was the most prominent in sepsis group.
Table (2): EIA results of serum levels of DcR 3 in sepsis, SIRS and control group.

<table>
<thead>
<tr>
<th>DcR3</th>
<th>Sepsis (N=25)</th>
<th>SIRS(N=27)</th>
<th>Control (N=28)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range</td>
<td>0.814 − 17.985</td>
<td>0.734 − 3.246</td>
<td>0.366 − 0.814</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>5.59 ± 4.79</td>
<td>1.67 ± 0.66</td>
<td>0.541 ± 0.11</td>
</tr>
<tr>
<td>F test</td>
<td></td>
<td>8.588</td>
<td></td>
</tr>
<tr>
<td>P value</td>
<td></td>
<td>0.001*</td>
<td></td>
</tr>
</tbody>
</table>

Table (3): Relationship of DcR 3 serum levels to PCR results

<table>
<thead>
<tr>
<th>DcR3</th>
<th>PCR positive Blood culture positive( No 22)</th>
<th>PCR positive Blood culture negative(No 3)</th>
<th>PCR negative Blood culture negative(No 27)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range</td>
<td>0.814-17.985</td>
<td>2.282-3.842</td>
<td>0.734-3.246</td>
</tr>
<tr>
<td>Mean ±SD</td>
<td>5.93±5.02</td>
<td>3.07±0.781</td>
<td>1.67±0.66</td>
</tr>
<tr>
<td>F test</td>
<td></td>
<td>10.032</td>
<td></td>
</tr>
<tr>
<td>P value</td>
<td></td>
<td>0.001</td>
<td></td>
</tr>
</tbody>
</table>

Fig. (1): Correlation of serum levels of DcR 3 and TLC, ESR and CRP in sepsis group

There was non-significant correlations between serum level of DcR 3 and TLC (p value 0.181) and there were significant correlations between serum level of DcR and ESR (p value 0.001) and between DcR and CRP (p value 0.001) in SIRS group.

The patients were classified into sepsis and SIRS according to results of blood culture which were kindly provided by laboratory managers of the hospital. They reported 22/52 patients
(42.3%) with positive bacterial blood culture and 30/52 patients (57.7%) with negative bacterial blood culture.

Pathogens detected in the sepsis group were reported as follows:
Gram-negative bacteria:
- 16/22 cases (72.73%)
  - E-coli: 7 cases (31.8%)
  - Pseudomonas: 4 cases (18.2%)
  - Klebsiella: 3 cases (13.6%)
  - Proteus: 2 cases (9.1%)

Gram-positive bacteria:
- 6/22 cases (27.27%)
  - Coagulase positive Staph: 3 cases (13.6%)
  - Coagulase negative Staph: 3 cases (13.6%)

So in the present study, 3/52 (5.8%) were positive PCR while their blood culture was negative.

**DISCUSSION**

DcR3 is a tumor necrosis factor receptor superfamily member that was identified in 1998 by the search of genes with homology to the TNFR gene super family in Expressed Sequence Tag (EST) database.\[^4]\) DcR3 exerts pleiotropic roles as a decoy receptor by an anti-apoptotic activity and a non-decoy receptor by its direct immune-modulatory properties. Recent studies have shown that DcR3 is up-regulated and may be pathogenetically implicated in sepsis and diverse chronic inflammatory diseases.\[^13]\ (Devitt and Marshall 2011).

Sepsis is an infection-initiated systemic inflammatory syndrome with an estimated incidence of 18 million cases annually worldwide. Despite advances in intensive care and supportive technology, the mortality rate of sepsis still high and the incidence of severe postoperative sepsis trebled from 0.3% to 0.9%, reminding scientists and clinicians that it remains to be a major clinical challenge.\[^14]\)

In sepsis patients, the identification of causative pathogens through blood cultures is still the gold standard for the diagnosis. However, confirmation of pathogens by cultures is slow and it may yield false negative results and the fact that positive blood cultures can be found for only approximately 65% of these patients. These false negative blood cultures may be
attributed to wrong or insufficient blood sample inoculum, very low bacterial load early in sepsis, empirical or long-term antibiotic use prior to diagnosis. Some bacterial species, however, are difficult to isolate, or grow slowly in the laboratory due to stringent growth requirements or lack of automated equipment.\textsuperscript{[15]}

Therefore by using PCR primers that are targeted at conserved regions of rDNA, it is possible to design broad range PCRs capable of detecting DNA of almost any bacterial species in the laboratory specimens.\textsuperscript{[10], [11] and [16]}, had utilized 16S rRNA gene for diagnosis of sepsis directly from blood by using different primers with different sensitivities for detection of 16S ribosomal DNA gene by PCR.

In the present study 52 hospitalized patients were studied in the early 48 hours of admission with signs and symptoms suggestive of septicemia. We tested the presence of bacterial pathogen directly in the blood by amplification of 16S ribosomal DNA gene by PCR for the 52 patients. 25/52 (48%) were found PCR positive and 27/52 (52%) were found PCR negative.

There were 3/52 (5.8%) PCR positive patient's specimens. When referred to their blood culture results – kindly provided by their hospitals laboratories- were found to be blood culture negative. Meanwhile the clinical parameters of those three patients, together with their high CRP, ESR and TLC results suggested bacterial sepsis. In the current study PCR results were validated regarding sensitivity, specificity, positive predictive value and negative predictive value as 100%, 90%, 88% and 100% respectively as referred to blood culture results.

Similar finding was reported by\textsuperscript{[10]}, who tested presence of bacterial sepsis in 100 neonate by blood culture and PCR amplification of 16S ribosomal DNA gene utilizing the same primers used in the present study. In their study blood culture yielded positive results in 9/100 (9%) of the study group and had a sensitivity of 69.2% for diagnosis of neonatal sepsis. PCR amplification was positive in 13/100 cases (13%). This included 4 cases with positive PCR but negative blood culture. All blood culture positive cases were PCR positive. They reported that PCR sensitivity was 100%, specificity was 95.6% and negative predictive value was 100%.
However,\cite{11}, studied 180 neonates and evaluated the presence of bacterial DNA in blood samples by PCR amplification of the DNA region encoding 16 rRNA utilizing the same primers used in the present study. The sensitivity, specificity, positive predictive value and negative predictive value of PCR in relation to blood cultures were 66.6%, 79.4%, 78.7% and 67.5% respectively.

Another study\cite{17} studied 48 patients with suspected sepsis were included, 31 patients were diagnosed with sepsis clinically, only 6 of these had a positive blood culture. Analysis of blinded blood samples from the 48 patients revealed 10 samples PCR positive compared to blood culture the diagnosis of bacterial proven sepsis by PCR revealed 66.7% sensitivity, 87.5% specificity, 95.4% positive and 75% negative predictive value. However in diagnosis of neonatal sepsis\cite{18}, found that PCR assay yielded a sensitivity of 79%, a specificity of 90%, a positive predictive value of 59% and a negative predictive value of 96% in relation to blood culture.

Ali, et al (2002)\cite{9} studied also infective endocarditis by comparison of blood culture with amplification of 16S ribosomal DNA gene by PCR utilizing the same primers used in the present study. They found that 8/50 (16%) were blood culture positive and 10/50 (20%) were positive by PCR. The sensitivity, specificity, PPV and NPV of PCR in relation to blood culture were 100%, 84%, 80% and 100% respectively.

For the foreseeable future, culture will not be superseded by PCR- based testing due to the requirement for purified culture isolates in antimicrobial susceptibility testing. However, an amplification assay could reduce significantly the overall medical costs to the health care system and concerning the time consumed to detect sepsis, especially for high risk patients when rapid diagnosis is mandatory and lifesaving and according to international policy and procedures, the accepted turnaround time (TAT) for BC is 72-120 hours, while PCR utilizes short turnaround time 6-12 hours.\cite{19}

The objective of this study was to evaluate the usefulness of measuring serum level of DcR 3 by enzyme immunoassay(EIA) within the first 48 hours of hospitalization to test its efficacy as an early and specific marker for prediction of bacterial sepsis, in addition to amplification of 16S ribosomal RNA as rapid diagnostic tool for sepsis.
In the present study, the average DcR3 level was 10.35 and 3.09 folds than the control group in sepsis and SIRS groups respectively. DcR3 concentrations between sepsis (5.59 ± 4.79ng/ml) and SIRS (1.67 ± 0.66 ng/ml) showed a 3.35 fold difference which was significant \((p <0.001)\). Therefore, There were significant increases of DcR3 in both groups compared to Normal and the most prominent increase was in sepsis group. The results of our study revealed that serum level of DcR 3 was an accurate biomarkers in differentiating sepsis from SIRS.

Our results are in agreement with the study of[20] who found that DcR3 concentrations in sepsis (10.46 ± 1.46 ng/ml) and SIRS (2.06 ± 0.33 ng/ml) showed a 5.1-fold difference which was highly significant \((p <0.001)\). Hou et al, (2012)[21] also agreed with our results as they found that the serum DcR3 was significantly increased in sepsis patients compared with SIRS patients and healthy controls (6.11±2.58 ng/ml vs 2.62±1.46 ng/ml, and 0.91±0.56 ng/ml, respectively, p<0.001). In addition, the DcR3 exhibited a positive correlation with the APACHE II("Acute Physiology and Chronic Health Evaluation II") score, a most commonly used index for the severity of sepsis.

These results were also complementary to findings of another study done by[22] also reported DcR3 as a valuable predictor of adverse outcome in patients with ARDS. Only DcR3 serum concentration discriminated those patients with multiple organ failure and mortality within 28-d from survivors, as the predictive value of DcR3 was evident from the first week of ARDS.

Yong et al, (2014)[23] who demonstrated that DcR3 levels were elevated in CSF of patients with bacterial meningitis (0.646 (0.229–1.514) ng/mL than those with non-bacterial meningitis 0 (0–0.192) ng/mL(p < 0.001). They recommended that detection of DcR3 level in CSF provides convenient and high accurate biomarker for diagnosis of bacterial meningitis. Recently[24] demonstrated that DcR3 protein treatments significantly improved survival in septic mice by 50% \((p <0.05)\) by suppressing the inflammatory response and lymphocyte apoptosis. They recommended that DcR3 protein may be useful in treatment of sepsis.

It is well known that, WBC is an important indicator of bacterial sepsis. In the present study the average WBC counts in both groups were higher than the reference range of 4,000~11,000 cells/ìl and there was significant difference in the cell counts between sepsis and SIRS groups. \((P = 0.001*)\).
Finally, in the present study, there were significant correlations between serum level of DcR3 and TLC (p value 0.001), ESR (p value 0.001) and CRP (p value 0.001) in sepsis group.

CONCLUSION AND RECOMMENDATION

Based upon our results, measurement of serum level DcR3 by quantitative sandwich EIA provides a noninvasive, specific biomarker for prediction of bacterial sepsis. Nevertheless the amplification of 16S ribosomal DNA gene by PCR implements broad range and time saving diagnostic technique complementary to culture based protocols.

An integrated laboratory analytic parameters of nonspecific indicators (ESR, CRP and WBC) together with specific serum marker DcR3 may serve as cost effective and first line panel predictors of sepsis in high risk patients in parallel with pathogen detection.

Rapid diagnosis of sepsis has a major impact on the clinical course, management and outcome of high risk ICU patients, it is also a challenge for both clinician and laboratory medical providers.

REFERENCES


