Value of 16S rRNA Gene Amplification for Early Detection of Bacteremia In Immunocompromised Patients

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Abstract: Background: Treatment of cancer is usually complicated by episodes of fever and neutropenia. Life-threatening infections, especially bacteremia, are common complications in those patients resulting in high morbidity and mortality. Administration of prophylactic or empirical antibiotics has reduced the mortality but decrease the sensitivity of diagnostic tests. So, there is a need for a rapid and accurate diagnostic tool to save those patients. **Aim of the work:** Our aim was to evaluate the use of more rapid diagnostic tool based on the detection of 16SrRNA gene by PCR with blood culture by using BACTEC 9240 for detection of bacteremia in immunocompromised patients with episodes of fever and neutropenia. **Patients and Methods:** Ninety-two immunocompromised patients who developed fever whilst neutropenic were included. Two blood samples were taken from each patient during the episode, the first for blood culture and the second for 16SrRNA gene detection by PCR. **Results:** Forty-five out of the 92 blood samples (49%) were positive for bacterial growth as detected by BACTEC 9240. Of these isolated bacterial species nine isolates were Gram-positive (20%) and 36 isolates (80%) were Gram-negative. These isolates were 4 Gram-positive species and 10 Gram-negative species .The most commonly isolated species were *E.coli* (17.8%), *K.pneumonie* and *P.auerоginosa* (13.3% each) and both *S.aureus* and *K.oxytoca* (9% each).The detection time of positive BACTEC 9240 cultures ranged from 1 to 6 days. Forty-one out of the 92 specimens (45%) were positive for the presence of bacteria by16SrRNA gene detection by PCR. Four bacterial species which were detected by blood culture were negative by PCR. The Kappa coefficient was 96%, which shows good agreement and indicates that 16SrRNA PCR gene detection can be used as an alternative method to blood culture for the detection of bacteremia. The method showed high specificity [100 % (91- 100)], sensitivity [91% (78 - 97)], accuracy (96%), PPV [100% (89 - 100)] and NPV [92% (80 - 97) %]. **Conclusion:** Analysis of 16SrRNA gene is a rapid and powerful tool for identifying pathogens especially in immunocompromised patients with life-threatening infections, and it is recommended to be applied in the clinical laboratory but it is still relatively expensive. So by decreasing the cost, this technology is likely to be applied in the clinical setting.
[Maha I. El Zaafarany, Tawfik R. Elkhodary, Muhammed I. El Zaafarany, SehamM.Seif, Maggie R. Mesbah and Wafaa M. Badawy. **Value of 16S rRNA Gene Amplification for Early Detection of Bacteremia in Immunocompromised Patients.** *J Am Sci* 2014;10(1):165-172]. (ISSN: 1545-1003). http://www.jofamericanscience.org. 26

Keywords: 16S rRNA Gene Amplification , Bacteremia, Immunocompromised Patient

1.Introduction:

They have been increase in the number of patients who are immunocompromised as a consequence of a primary or secondary immune deficiency disorder or from the use of agents that depress one or more components of the immune system. (1)

Immunocompromised host has an alteration in phagocytic, cellular or humoral immunity that increases the risk of an infectious complication or an opportunistic process such as a lymphoproliferative disorder or cancer. (2)

Patients may also be immunocompromised if they have an alteration or break of their skin or mucosal defense barriers that permits microorganisms to cause either local or systemic infections e.g. indwelling catheter, burn and dialysis patients. (1)

Episodes of fever and neutropenia are common complications of treatment for cancer and dialysis patients. The most important risk factor for infection is the degree of neutropenia although other risk factors include disturbance of physical defense barriers and alteration in microflora. (3)

Virtually any organism can cause serious infections in immunocompromised host, including commensal bacteria and organisms of low virulence that do not cause infection in the immunocompetent host. (4)

Bacteremia continues to result in significant

morbidity and mortality, particularly in patients who are immunocompromised [5]. It is also one of the major types of nosocomial infections in most of health care centers. [6]

It has been estimated that the crude mortality rate in patients with bacteremia ranges from 25-50% with close to one third of deaths being directly attributable to these infections. [7]

Broad spectrum antibiotics are administered to patients suspected to have blood stream infections that are awaiting diagnosis that depends on blood culture analysis. Significant delays in identification of pathogens can result primarily due to the dependence on growth-based identification systems. [8]

Currently, blood culturing is considered the gold standard for diagnosing bacteremias. However, even blood culturing techniques can have unacceptably low sensitivity due to intermittent seeding of low numbers of bacteria within the blood, the presence of antibiotics in patient sera and the fastidious characteristics of certain species not supported by the standard blood culture system. [9]

Also, no pathogen could be identified in 35-60% of episodes of fever and neutropenia. [6]

Riikonen*et.al.* investigated blood culture negative febrile neutropenic episodes and found serological evidence of bacterial infection in 35% of these infections.[10]

Molecular techniques such as PCR have been used successfully to identify a wide range of organisms. Unlike culture types of assays they don`t depend on growth of an organism for detection. It has the potential for excellent sensitivity and a shorter turnaround time than those of culture-based protocols. [9]

Several features of the 16S rRNA gene make it an important phylogenetic tool and hence a useful target for clinical identification. First, it is present in all bacteria thus it is a universal target for bacterial identification. Second, its function has remained constant over a long period. Finally, 16S rRNA gene is large enough (approximately 1500bp) for relevant sequence information. [11]

Aim of the work:

Our aim was to evaluate the use of more rapid diagnostic tool based on the detection of 16SrRNA gene by PCR with blood culture using BACTEC 9240 for detection of bacteremia in immunocompromised patients with episodes of fever and neutropenia. The sensitivity, specificity, accuracy, positive predictive value (PPV) and negative predictive value (NPV) of both methods were determined.

2.Patients and methods:

This study was conducted over a period of 10 months from February 2010 to October 2010 on 92 immunocompromised patients.

1-Patients

1.1 Selection of patients

It included 92 immunocompromised patients who were admitted to MUH and had episodes of fever and neutropenia during the period of study. The first group included 42 patients admitted to Oncology centre and received standard chemotherapy. The second group included 30 patients from bone marrow transplant unit (BMT) and the third group included 20 patients who had undergone liver transplantation.

1.2 Definition of episode of fever and neutropenia

An episode of fever and neutropenia was defined by a temperature of 38°C on 2 occasions in the presence of an absolute neutrophil count of less than $1x10^9$/L. No patients were excluded because of prior antibiotic therapy. [3]

1.3 Blood samples:

Blood samples were collected from the 92 patients (42 oncology patients, 30 patients from BMT unit and 20 patients from liver transplantation unit) each of them was sampled once. Two blood samples were collected from each patient at the start of a febrile episode; the first blood sample was used for blood culture and the second for PCR. Ten ml of blood for culture was collected from either central venous catheter (CVC) or from a peripheral vein in the absence of CVC and was inoculated in to aerobic and anaerobic blood culture bottles using an automated continuous- monitoring blood culture system, BACTEC 9240(Becton Dickinson, Sparks, Md.) The second sample was 5 ml EDTA anticoagulated blood. The blood culture bottles were gently rotated to mix the blood and the broth. The blood specimens in EDTA-treated tubes were also mixed well by inverting 8 - 10 times.

2-Methods:

2.1 Blood culture processing

Blood culture vials were inoculated in an automated continuous- monitoring blood culture system, BACTEC 9240(Becton Dickinson, Sparks,Md.). The BACTEC system utilizes a fluorescent sensor for detecting microorganisms and relies primarily on the detection of CO_2 produced by actively metabolized microorganisms. The bottles were incubated immediately upon receipt in the microbiology laboratory in accordance with the manufacture's recommendation. Subculture was done from positive culture vials on blood, chocolate and MacConkey agar media. The culture bottles that did not show any sign of bacterial growth after seven days of incubation were reported as negative. Appropriate biochemical reactions were done to identify the species. [12]

2.2 Detection of16SrRNA gene by PCR

Bacterial DNA extraction:

Bacterial genomic DNA was extracted using QIAamp DNA blood mini kit (Qiagen, Germany) following the manufacture's protocol. The resultant DNA extract was frozen at -20°C until use.

Primers

Universal broad-range PCR was carried out with the primers DG74 (5`-AGGAGGTGATCCAACCGCA-3`) and 65ab (5`- AACTGGAGGAAGGTGGGGAY-3`); Gram-positive-specific PCR was carried out with the primers DG74 and 143(5`-GAYGACGTCAARTCMTCATGC-3`); and Gram-negative-specific PCR was carried out using the primers DG74 and 68d (5`-AYGACGTCAAGTCAAGTCMTCATGG-3`) (Promega). [13,14]

Polymerase Chain Reaction Amplification

The PCR reactions were performed in a total volume of 50 µl. Two µl of DNA, 1 µl of sense primer and 1 µl of antisense primer were added to 46 µl of master mix. containing 3 µl MgCl2 solution (1.5 mm), 5 µl of 10X PCR buffer 1 µl dNTPs mixture (10 mM/ml), 0.4 µl Ampli Taq DNA polymerase (5units/µl) all in 36.6 µl distilled water (Finnzymes, Espoo, Finland).The DNA extracted from blood samples was added to the PCR mixture at 75° C within a laminar air flow and the reaction mixture was overlaid with two drops of paraffin oil. The initial denaturation was done at 95°C for 5 min. A total of 30 cycles of PCR were performed using a thermal cycler(MJ Research, Inc., USA) consists of a denaturation step for 30 seconds at 95°C, an annealing step for 30 sec at 65°C, and an extension step for 2 min. at 72°C.Final extension step for 5 minutes at 72°C. Universal broad range PCR primers were initially used on the whole blood. The expected amplicon was 400 bp. Specimens with negative results were repeated twice. Specimens with positive results were further subjected to Gram-type specific primers to identify Gram-positive and Gram-negative bacteria. Both sets of primers yielded amplicons of 400bp.

Controls for PCR

For positive controls, blood from a healthy person was separately inoculated with two pure colonies each of *S.aureus* and *E.coli*. The tubes were incubated for 2h at room temperature and specimens were processed as described earlier. Likewise, blood from the healthy individuals was used as negative control.PCR was carried out on blood samples using primers for the human β-globin gene. This served as an internal control to rule out the presence of PCR inhibitor. The reaction mixture consisted of 1X PCR buffer,1.5Mm $Mgcl_2$, 200µM deoxynucleotide mix, 0.02 U/µl Taq DNA polymerase, 0.5µM each of BGF (5`-GCCAGTGCCAGAAGAGCCAA-3`) primers and BGR(5`-TTAGGGTTGCCCATAACAGC-3`) primers [15]and DNA template. The PCR conditions were as follows: an initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 30 sec, 72°C for 1 min and final extension at 72°C for 5 min. The expected amplicons were 500 bp in size.

Agarose gel electrophoresis

The PCR products together with the controls were visualised by ethidium bromide staining after electrophoresis in a gel containing 2% agarose with DNA standard marker: Φ X 174 (HaeIII) digest marker (Promega).

2.3 Statistical analysis [16]

The statistical analysis of data done by using excel program and SPSS program statistical package for social science version 10.The description of the data done in form of mean ±SD for quantitative data, frequency and proportion for qualitative data. The analysis of the data was done to test statistical significant difference between groups. For quantitative data student t-test was used to compare between 2 groups. Chi-square test was used to compare qualitative data. *P* is significant if < or = 0.05 at confidence interval 95%.

3- Results:

Study population

Our study which was conducted over 8 months from February to October 2010.It included 92 immunocompromised patients who were admitted to MUH and had episodes of fever and neutropenia. The first group included 42 patients admitted to Oncology centre. The second group included 30 patients from BMT unit and the third group included 20 patients from the liver transplant unit. The study population consisted of 43 (46.7 %) males and 49(53.3 %) females, with ages ranged from 20-87 (*46.8±16.*).Evaluation of the inclusion criteria showed that the body temperature of the study population ranged from 37.6-39.1°C (*37.8±0.56*). The leukocyte counts of the patients ranged from 300-1000/mm^3 (mean of700±*125/*mm^3).Fever duration was significantly longer in episodes that were PCR positive (T=3.96, *P* 0.044*).

BACTEC 9240 blood culture results

Forty-five out of the 92 blood samples (49%) were positive for bacterial growth as detected by BACTEC 9240.Each sample represented different

patient and yielded only single isolated species. Of these positive (45) blood cultures, 27(60%) were detected from patients admitted in the Oncology Unit,12 (26.7%) from BMT Unit and 6(13.3%) from liver transplant Unit.

Of these 45 isolated bacterial species nine isolates were Gram-positive (20%) and 36 isolates (80%) were Gram-negative. These isolates were 4 Gram-positive species and 10 Gram-negative species (Table 1).The most commonly isolated species were *E.coli* (17.8%), *K.pneumonie* and *P.aueroginosa* (13.3%) each and both *S.aureus* and *K.oxytoca* 9% each (Table 2).The time of positive BACTEC cultures ranged from 1 to 6 days.

Table (1): Bacterial detection by BACTEC 9240 and PCR FOR 16SrRNA.

Method	Positive results	Gram positive	Gram negative
BACTEC 9420	45/92 (49%)	9/45 (20%)	36/45 (80%)
16SrRNA-PCR	41/92 (45%)	8/41 (19.5%)	33/41 (80.5%)

X2=0.35 P=0.55

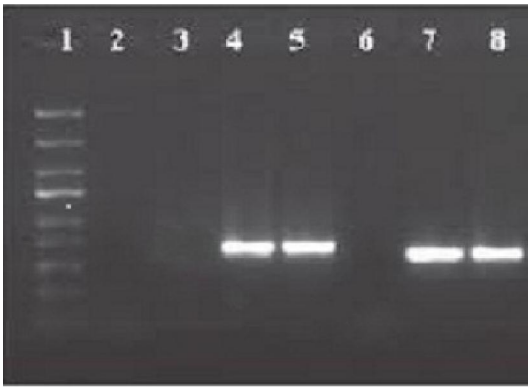
Sensitivity : 91% (78 - 97).
Specificity : 100% (91- 100).
Accuracy : 96% (89 - 99).
Positive predictive value (PPV): 100% (89 - 100).
Negative predictive value (NPV): 92% (80 - 97).

Table (2): Summary of positive specimens by both BACTEC 9240 blood culture and 16SrRNA gene detection by PCR.

Sample	**BACTEC 9240 day +ve**	**BACTEC 9240 +ve blood culture**	**PCR +ve**
16,23,54	2nd	*S.aureus*	Positive
75	3rd	*S.aureus*	Positive
2	3rd	*S.epidermidis*	Positive
39	4th	*S.epidermidis*	Positive
42	3rd	*S.viridans*	Positive
33	2nd	*E.fecalis*	Negative
47	3rd	*E.fecalis*	Positive
17,45,68	2nd	*E.coli*	Positive
5,20	4th	*E.coli*	Positive
22,51,90	4th	*E.coli*	Positive
11,26,55	1st	*K.pneumonie*	Positive
48,73	2nd	*K.pneumonie*	Positive
81	3rd	*K.pneumonie*	Positive
9	1st	*P.aueroginosa*	Negative
36,74	2nd	*P.aueroginosa*	Positive
57,63	3rd	*P.aueroginosa*	Positive
77	1st	*K.oxytoca*	Negative
32,50,62	3rd	*K.oxytoca*	Positive
7,49,86	5th	*P.mirabilis*	Positive
29,71	2nd	*S.maltophilia*	Positive
58	3rd	*S.maltophilia*	Negative
15	3rd	*A.baumannii*	Positive
44	2nd	*A.baumannii*	Positive
28,61	2nd	*E.cloacae*	Positive
70	3rd	*P.vulgaris*	Positive
69	5th	*B.fragilis*	Positive

Table (3):The percentage of different isolated bacteria from blood of bacteremic patients by BACTEC 9240.

Bacterial species	Incidence	
	No	%
1-Gram positive	9	(20)
S.aureus	4	9
S.epidermidis	2	4.4
E.fecalis	2	4.4
S.viridans	1	2.2
2-Gram negative	36	(80)
E.coli	8	17.8
K.pneumonie	6	13.3
P.auerogenosa	6	13.3
K.oxytoca	4	9
P.mirabilis	3	6.7
S.maltophilia	3	6.7
A.baumannii	2	4.4
E.cloacae	2	4.4
P.vulgaris	1	2.2
B.fragilis	1	2.2



Graph (1) the ethedium bromide staining pattern of agarose gel 2% electrophoresis lane 1 represent marker OX174/Hae III DNA ladder marker, lane 3,6 negative sample, lane 4, 5 500bp positive control , lane 2 negative control, lane 7, 8 400pb positive sample

Comparison of 16SrRNA gene detection by PCR with BACTEC 9240 blood culture

Forty-one of the 92 specimens (45%) were positive for the presence of bacteria by16SrRNA gene detection by PCR (Table 3).Four bacterial species which were detected by blood culture, were negative by PCR. The Kappa coefficient was *96%* which shows good agreement and indicates that 16SrRNA PCR gene detection can be used as an alternative method to blood culture for the detection of bacteremia. The method showed high specificity [100 % (91- 100)], sensitivity [91% (78 - 97)], accuracy (96%) , PPV [100% (89 - 100)] and NPV[92% (80 - 97) %].

4.Discussion:

The past decades showed an increase in the number of immunocompromised patients. Fever is the principal and sometimes the only manifestation of serious infection in those patients. Also, neutropenia represents one of the most important clinical conditions associated with life-threatening infections in those patients. [(1)]

The incidence of bacteremia has increased over the past decades with an overall in-patient mortality of 20% which can reach 90% in patients with septic shock and organ failure. [(17)] Detection and identification of bacteria from blood of patients is

one of the most important roles of clinical microbiology laboratory. [18] However, the assay requires incubation, sub-culturing and biochemical and/or immunologic tests to identify the bacterium. [19]Also, detection of pathogens in a large proportion of episodes of fever associated with neutropenia remains negative when using standard culture technique. [10]

Molecular assays based on nucleic acids have been shown to have greater sensitivity and specificity in numerous studies in identification of bacteria in different clinical samples. [19]

Our aim was to evaluate the use of more rapid diagnostic tool based on the detection of 16SrRNA gene by PCR with blood culture using BACTEC 9240 for detection of bacteremia in immunocompromised patients with episodes of fever and neutropenia. Thisstudy was conducted over a period of 10 months from February 2010 to October 2010 on 92 immunocompromised patients with episodes of fever and neutropenia(42 from Oncology Unit,30 from BMT unit and 20 liver transplant unit).

Our results showed that positive blood cultures by BACTEC 9240 were detected in 49% of blood samples (Table 1). Of those positive blood cultures, 60% were detected from the first group, 26.7% from the second group and 13.3% from the third group. The detection time for bacteremia ranged from 1 to 5 days. Similarly, Qian*et al.* reported that although BACTEC has high sensitivity but it requires longer time for identification of microorganisms. [18]

Of these positive blood cultures Gram-positive bacteria represented 20% of isolates including 4 species: *S.aureus* (9%), *S.epidermidis* and *E.fecalis* (4.4% each) and *S.viridans* (2.2%).This had been reported by Phillip and Pizzo,1999 who reported that Gram-positive organisms specially coagulase negative Staphylococci have emerged as leading cause of acute bacterial infections associated with fever and neutropenia in patients in United States and Europe which may be partly due to the increase use of indwelling intravenous access devices. [1]

Gram-negative species were isolated from 80% of positive blood cultures. There were 10 isolated species with the most common isolated species were: *E.coli* (17.8%),*K.pneumonie* , *P.aueroginosa* (13.3% each), and *K.oxytoca* (8.9%). The other isolated species were: *P.mirabilis, S.maltophilia* (6.7% each), *A.baumannii*, *E.cloacae* (4.4% each) and lastly *P.vulgaris*and *B. fragilis* (2.2% each).It has been reported that in developing countries Gram-negative organisms causing infections in immunocompromised host with neutropenia including *E.coli,K.pneumonie* and *P.aueroginosa* still predominate with a pattern of infection similar to that in the USA and Europe in 1960 and 1970. [1].Although blood culture presents many advantages and high accuracy for sepsis or other life-threatening infections but a more rapid diagnostic tool is preferable. [9]

Using 16SrRNA gene detection by PCR, positive results were obtained from 41% of blood samples. Microorganisms from four samples identified as *E. fecalis*, *P.aueroginosa*, *K.oxytoca* and *S.maltophilia* by BACTEC9240 were not detected, but the internal controls for the human β-globin gene did not produce amplicons in these samples, which may be due to the presence of certain inhibitory factors in the blood samples such as hemoglobin [20] , immunoglobulin G [21] and anticoagulant . [22]

These results indicates that 16SrRNA PCR gene detection can be used as an alternative method to blood culture for the detection of bacteremia withthe Kappa coefficient was 96% , which shows good agreement and indicates that 16SrRNA PCR gene detection can be used as an alternative method to blood culture for the detection of bacteremia. The high specificity [100 % (91- 100)] , sensitivity [91% (78 - 97)], accuracy (96%), PPV [100% (89 - 100)] and NPV [92% (80 - 97) %] in addition to the more rapidity for diagnosis support its utilization as an alternative to the culture method. Similarly, Ley *et al.* whose study was designed to assess the extent to which the use of PCR with euobacterial primers could improve the diagnostic yield and their results suggested that molecular techniques could augment cultural methods in the diagnosis of bacteremia in patients who have been treated with antibiotics. [3] Also, Valle *et al.* who compared four methods for detection of bacteremia, whole blood culture, buffy coat culture, buffy coat 16SrRNA and whole blood 16SrRNA PCR utilizing broad range16SrDNA primers and Gram-specific primers and reported that the three methods showed very high sensitivity, specificity, positive and negative predictive values when compared to whole blood culture. [17]

A significant finding in our study is the isolation of *P.aueroginosa, S.maltophilia* and *A.baumannii* which represent 23.3% of all isolates from cases of fever with neutropenia and these organisms are considered from emerging pathogens causing serious nosocomial infections. The high occurrence of these organisms in the present study reflects the persistence and transmissibility of these organisms specially among those vulnerable patients. [23]

Conclusion:

Analysis of 16SrRNA gene is a powerful mechanism for identifying pathogens especially in immunocompromised patients with life-threatening infections, and it is recommended to be applied in the clinical laboratory but it is still relatively expensive. So, by decreasing the cost, this technology is likely to be applied in the clinical setting.

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12/22/2013