Nested multiplex PCR for detection of bacterial and fungal blood stream infections in patients with hematological malignancies

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Abstract

Introduction: Bloodstream Infections (BSIs) are a main cause of life-threatening complications among patients with cancer.

Methodology: This study aimed to identify microbial pathogens causing BSI in febrile neutropenic patients with hematologic malignancy and compare the results of conventional blood culture with a nested multiplex real time PCR assay done directly on whole blood samples. The nested multiplex PCR was based on 16S rDNA and 18S rDNA sequence-specific primers; hence, it allowed the identification of most species of bacteria and fungi.

Results: Forty adult patients with febrile neutropenia, admitted at Hematology ward of Ain Shams University Hospitals, were included in this study. Each patient was subjected to conventional blood culture and nested multiplex PCR. Blood culture was positive in 19 patients (47.5%). About 68.4% of the positive cultures were monomicrobial, while 31.6% were polymicrobial. A total number of 26 isolates were grown from positive cultures; Staphylococcus aureus was the most common (30.8%), followed by Klebsiella pneumoniae (19.2%). Regarding nested PCR, positive results were detected in 37/40 patients (92.5%) which was statistically significantly higher than that of blood culture. Eighteen samples that tested negative by culture were positive using the molecular approach. The agreement between the two approaches was 55%.

Conclusion: nested multiplex real time PCR can be a promising tool in order to achieve rapid diagnosis in cancer patients clinically suspected of BSIs. Its utilization could affect the choice of antimicrobial treatment whether bacterial or fungal and, therefore avoid unnecessary use of antimicrobials.

Key words: bloodstream infections; nested multiplex PCR; hematological malignancy; diagnosis.

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Introduction

Patients with hematologic malignancies (HM) are at high risk of infectious complications, blood stream infections (BSIs) are the most severe among these [1]. This is not only because of the malignancy itself, but also because of neutropenia induced by intensive chemotherapy and its cytotoxic effect on the cells lining the gastrointestinal tract [2].

The epidemiology of microbial pathogens and antimicrobial resistance may differ by geographical region [3]. The most common bacteria in BSIs are coagulase negative staphylococci, Enterococcus spp. and Escherichia coli. Fungal BSIs are rapidly increasing over the last decades mostly due to increased cases of immune suppression. Candida spp. is the most commonly isolated fungal pathogen from BSIs. [4].

Epidemiological data from resource limited countries as Egypt are sparse. Very few countries of low and middle income have national health care associated surveillance program [5].

The reference method used for detection of pathogens in blood of septic patients is blood culture followed by conventional identification methods. Benefits of such method are its simplicity and low cost. Its weakness is that it is time-consuming, and possesses low sensitivity, which leads to obtaining microbial growth in only 15–20% of the cultures. Detecting microbes in blood is extremely difficult regarding their relatively small number, additionally limited by formerly applied antibiotic treatment [6].

Molecular methods offer a new and rapid alternate to conventional culture, minimizing the time for diagnosis. Moreover, these techniques showed less affection by the administration of empirical antimicrobial therapy [7].
Broad-spectrum PCR assays, allowing more universal detection of microbes would have a substantial impact on the management of patients with suspected infections [8]. A nested multiplex real time PCR assay developed by Gosiewski et al. can simultaneously detect DNA of bacteria and fungi in blood by two sequential amplification reactions; which raises the sensitivity of detection by two orders of magnitude [9].

This study aimed to identify microbial pathogens causing BSIs in febrile neutropenic patients with HM at Ain Shams University hospital and compare the results of conventional blood culture with a nested multiplex real time PCR assay done directly on whole blood samples.

Methodology

This is an observational cross-sectional study conducted during the period between June and November 2018. The study was approved by the Hospital Ethics Committee of Ain Shams University Hospitals, approval number FMASU MD 73/2018 and an informed consent was taken directly from the patients or their relatives for sample collection.

The study enrolled 40 adult patients diagnosed with HM and hospitalized at hematology ward of Ain Shams University hospitals. The patients presented with febrile neutropenia defined according to the Infectious Diseases Society of America: as a single oral temperature of ≥ 38.3°C or a temperature of ≥ 38.0°C sustained over a one-hour period. Neutropenia is usually defined as an absolute neutrophil count (ANC) < 1500 cells/mm³ [10]. All patients had high CRP levels ranging from 8.2mg/L to 320mg/L. Confirmation of bloodstream infection was done according to CDC/NHS guidelines [11]. This was carried by conventional blood culture and nested multiplex PCR.

Blood culture

Two blood samples were withdrawn for each patient from two different peripheral sites under complete aseptic conditions, one 16 mL and the other 18 mL (2 mL were used for PCR). Samples were collected before administration of empirical antibiotic therapy. If antibiotics were already administered, blood was drawn just before the next dose was given. The blood samples were immediately inoculated into two sets of blood culture bottles; each set comprises 2 adults 70 mL HiSafe Dual Blood Culture bottles (Himedia, Mumbai, India), 8 mL each. A sterile venting needle with a membrane filter was used to ventilate one of the two culture bottles meant to be incubated under aerobic conditions. For anaerobic cultures, the bottles were not vented. The bottles were incubated at 37° C for seven days [12].

Microbial growth was denoted by growth on solid phase. Positive samples were sub-cultured on suitable media, and identification of the isolated organisms was done according to Tille [13].

Common skin contaminants (e.g. coagulase negative staphylococci) were considered significant only if they were found in two consecutive blood culture samples. BSI was defined as polymicrobial if two or more microorganisms were isolated from blood cultures [14].

| Table 1. Primers used in nested multiplex PCR according to Gosiewski et al. [9] |
|---------------------------------------------------------------|
| **First PCR run (reaction I)**                               | **Nucleotide sequences (5’–3’)**                           |
| For bacterial infection:                                     |                                                             |
| EXT_BAC_F                                                     | kGCCGrACGGGTGAGTAA                                         |
| EXT_BAC_R                                                     | CGCATTTCAACGGCA                                            |
| For fungal infection:                                        |                                                             |
| EXT_FUN_F                                                     | AATTGACGGAAGGGCACC                                         |
| EXT_FUN_R                                                     | TTCTCGTTGAAGAGCAA                                          |
| **The nested amplification (reaction II)**                   |                                                             |
| For bacterial infection:                                     |                                                             |
| GN/GP_ F                                                      | GACTCTACGGGAGGC                                           |
| GN/GP_ R                                                      | GCGGCTGCGGCAC                                             |
| For fungal infection:                                        |                                                             |
| FUN_F                                                         | TTTGTTGAGTGATTGTCTGCT                                       |
| FUN_R                                                         | TCTAAGGGCATCACAGACCTG                                       |
| **β-actin gene**                                             |                                                             |
| F                                                             | 5’GCCAGTGCCAGAAGAGGCA                                         |
| R                                                             | 5’TTAGGGTGGCCATAACGACG3’                                    |

EXT-BAC: external primer for bacteria detection; EXT-FUN: external primer for fungi detection; F: forward; R: reverse.
Nested multiplex PCR

Microbial DNA isolation and purification

Two mL of the collected blood were inoculated into a lavender-top EDTA-treated CBC tubes, stored at -80°C and were reserved for DNA extraction and nested multiplex PCR assay. Microbial DNA was isolated and purified using QIAamp DNA blood Mini Kit (Qiagen, Hilden, Germany), following the manufacturer’s protocols. The quality of DNA extraction was assessed by including beta actin gene as a house-keeping gene which validate proper DNA extraction. Samples which were negative for this gene were either excluded or subjected to DNA re-extraction. DNA quantity was measured by Eppendorf Bio-photometer to (5-12 μg of DNA for each sample). All the reactions were run in triplicates.

Microbial DNA amplification

Microbial DNA was amplified by a nested multiplex PCR assay according to Gosiewski et al. [9]. The used PCR was based on 16S rDNA and 18S rDNA sequence-specific primers; allowing the identification of most species of bacteria and fungi. The primer sequences are listed in Table 1. The first PCR run (amplification reaction I; external primers): DNA amplification was carried out using Hybaid HBPXE02110 PxE Thermal Cycler (Thermo scientific, Waltham, USA) under the following conditions: Initial denaturation at 94°C for 2 min, followed by 40 cycles at 94°C for 45 seconds, 46°C for 45 seconds, and 72°C for 1 minute and a final extension at 72°C for 2 minutes. The reaction was end point conventional PCR and amplified products were visualized using agarose gel electrophoresis stained with ethidium bromide and UV LUT-300D trans-illuminator (LABNICS, UK). A positive and negative controls and molecular size marker 1000 base pair (Bohringer Mannheim, Germany) were included with each run. The amplified product size was expected at 610bp for bacterial 16S rDNA and 440bp for fungal 18S rDNA (Figure 1).

The nested amplification reaction II (internal primers) was performed in a real time PCR reaction with melt curve analysis using StepOne™ Real-Time PCR System (Applied Biosystems, USA), following the cycling protocol: 2 minutes at 95°C, followed by 40 cycles of (30 s at 95°C and 60 s at 60°C). Internal bacterial and fungal primers were added to the master mix in separate wells following the protocol of GoTaq® qPCR Master Mix (Promega Cat. no. A6001/2). The amplification plot showed positive results while the machine is running. The cycle threshold (CT) value was recorded for each sample; positive ones have CT number while negative ones have no CT at all. A mean cycle threshold (Ct) of 32.03 was defined as cut-off value for positive PCR result to distinguish between true infection and contamination [15]. Specificity of the results was verified by melt curve analysis that is run automatically by the machine (Figure 2).

All samples were tested for beta actin gene in a real time PCR reaction with same conditions for the nested PCR. Beta actin gene was used as internal control in PCR amplification to allow detection of inhibition or failure of extraction. The gene is moderately expressed in white blood cells and[16].

Data analysis

Statistical Package for the Social Sciences (SPSS) of Windows computer program version 22 (USA) was used for analysis of data. Chi-square test was used for analysis of qualitative variables and was one-tailed (P-values are calculated). Cohen’s kappa was computed to evaluate concordance between blood culture results and
nested multiplex PCR results. A kappa value of 1 indicates perfect agreement and a kappa value of 0 no agreement.

**Results**

The demographic data of the forty patients included in the study were as follows; nineteen cases (47.5%) were males while twenty-one cases (52.5%) were females, their ages ranged from 18 to 69 years with mean ± SD of 37.18 ± 13.245. The clinical and laboratory data of the patients are summarized in Table 2. All patients received antimicrobial prophylaxis regimen that included a fluoroquinolone and oral triazole according to Infectious diseases society of America (IDSA) guidelines [17].

**Results of conventional blood culture**

Blood culture was positive in 19/40 cases (47.5%) while 21 (52.5%) yielded negative results. Out of the 19 culture positive patients; 13 (68.4%) had single pathogen, 5 (26.3%) had two pathogens and 1 (5.3%) patient had three pathogens with twenty-six isolates. Table 3 demonstrates the distribution of pathogens among the positive blood cultures. *Staphylococcus aureus* was the most common organism (30.8%), followed by *Klebsiella pneumoniae* (19.2%).

The distribution of pathogens in samples with polymicrobial infections were as follows: two samples yielded *Staphylococcus aureus* and *Candida tropicalis*, one sample yielded *Klebsiella pneumoniae* and *Enterococcus spp.*, one sample yielded *Staphylococcus aureus* and *Candida parapsilosis*, one sample yielded...
coagulase negative staphylococci and *Aspergillus* ssp., and one sample yielded *Klebsiella pneumoniae, Enterococcus* ssp., and *Candida parapsilosis*.

**Results of nested multiplex PCR**

PCR analysis of blood samples from the forty patients revealed 27(67.5%) were positive by the first PCR run. After nested amplification reaction II, 37 samples (92.5%) yielded positive results. Table 4 shows detailed results of the two PCR amplification reaction.

On comparing the results of nested PCR to conventional blood culture, we found a difference of high statistical significance (P < 0.001) between the results of the nested multiplex PCR assay and blood culture as shown in Table 5. Twenty-two patients had identical blood culture/nested PCR results, while 18 patients had positive nested PCR and negative bacterial blood culture. Slight agreement (55%) between conventional blood culture and nested multiplex PCR assay (k = 0.1366) was found.

**Discussion**

Bloodstream infection is a common complication in patients with cancer and results in significant levels of morbidity and mortality [18].

All patients included in the study received proper empirical antimicrobial therapy, patients with positive cultures received tailored therapy according to culture and sensitivity and patients with negative blood culture were reevaluated. Patients with pathogen-negative sepsis may represent an important opportunity for antimicrobial stewardship with the aid of molecular approaches, assuming that many of these patients received unnecessary antibiotic therapy.

In the present study, nearly half of the patients (47.5%) yielded positive culture results. Several studies reported positive blood culture in febrile neutropenic patients ranging from 30% to 60% [19-21]. These differences in positivity of blood culture result may be attributed to differences in blood volume withdrawn, blood culture techniques and exposure to antimicrobials [22].

We found that Gram positive cocci was predominant isolate (50%). Similar results were reported by other studies [23-25]. On the other hand, several other studies [26-29], found that Gram negative isolates were predominant. The predominance of Gram-positive organisms could be explained by intensive courses of chemotherapy causing damage of oro-intestinal mucosa, the frequent use of central venous catheters which contribute to developing skin-derived Gram-positive infections [30], and using antibiotic prophylaxis such as third generation cephalosporins and fluoroquinolones which are more active against Gram negative bacteria [31]. No anaerobic bacteria were isolated in the current study. This may be explained by

| Table 3. Distribution of isolated pathogens among positive blood cultures. |
|-------------------------------------------------|
| Isolates                                        | n (%) |
| Gram-positive cocci                             | 13 (50) |
| *Staphylococcus aureus*                         | 8 (30.8) |
| Coagulase negative staphylococci (CoNS)        | 3 (11.5) |
| *Enterococcus* ssp.                             | 2 (7.7) |
| Gram-negative bacilli                            | 7 (27) |
| *Klebsiella pneumoniae*                         | 5 (19.2) |
| *Escherichia coli*                              | 1 (3.8) |
| *Pseudomonas aeruginosa*                        | 1 (3.8) |
| **Fungi**                                       | 6 (23) |
| *Candida tropicalis*                            | 3 (11.5) |
| *Candida parapsilosis*                          | 2 (7.7) |
| *Aspergillus* ssp.                              | 1 (3.8) |
| **Total**                                       | 26 (100) |

| Table 4. Detailed results of nested multiplex PCR analysis of blood samples. |
|-------------------------------------------------|
| Positive only for bacteria | Positive only for fungi | Positive for both bacteria and fungi | Total positive samples | Negative for both bacteria and fungi |
|---|---|---|---|---|
| No | % | No | % | No | % | No | % | No | % |
| 1st run PCR | | | | | | | | | |
| 17 | 42.5 | 4 | 10 | 6 | 15 | 27 | 67.5 | 13 | 32.5 |
| Nested PCR | | | | | | | | | |
| 22 | 55 | 4 | 10 | 11 | 27.5 | 37 | 92.5 | 3 | 7.5 |

| Table 5. Results of nested multiplex PCR assay compared to conventional blood culture. |
|-------------------------------------------------|
| Nested multiplex PCR assay | Positive | Negative | Measure of agreement Kappa | P value |
|-----------------------------|---------|---------|---------------------------|--------|
| Conventional blood culture  | 19      | 0       | 0.1366                    | 0.000  |
|               | 18      | 3       |                           |        |
| Total           | 37      | 3       |                           |        |
the fact that these microorganisms are typically fastidious, slow growing and difficult to culture. The use of automated blood culture systems improves the detection of these microorganisms [32].

In our study, fungemia was diagnosed in 15% of patients (6/40). The prevalence of candidemia among patients with HM was found to vary widely between 1.6% and 22.9% depending on the patient profile studied, geographical location involved, and diagnostic criteria used [33]. Among fungal isolates in the current study, Candida tropicalis was the commonest isolate. This goes in accordance with Swati et al. and Wu et al., who reported that isolates of C. tropicalis are more frequently found among patients with cancer [34-35]. Patients with chemotherapy-induced neutropenia accumulate various risk factors for candidemia: they usually receive wide spectrum antibiotics for several days, they have serious gastrointestinal epithelial tissue dysfunction, and the use of vascular catheters for the infusion of chemotherapeutic drugs and antibiotics [36].

We found about 31.6% of culture positive samples were polymicrobial. One patient had positive blood culture for 3 pathogens, he suffered from severe neutropenia (absolute neutrophil count = 380 mm$^3$). The high rate of polymicrobial BSIs observed in our study is still within the reported range among different investigators (8% to 32%), and explained by neutropenia [37]. Being neutropenic is an independent risk factor for BSIs. Neutrophils are the prime cells against invading microorganisms, namely bacterial pathogens. [38].

Nested PCR yielded positive results higher than those obtained by first run PCR (92.5% vs 67.5%). This improvement in microbial detection by using nested multiplex PCR goes in accordance with Gosiewski et al.. The assay is based on applying a preliminary amplification procedure (I) so as to gain an opportunity to carry out detection of the presence of bacteria and fungi in the nested multiplex PCR system, which, in turn, allows considerable increase in the detection of bacteremia and fungemia. [11].

Our study showed that the nested multiplex PCR assay yielded higher results than blood culture, and no negative specimen by the nested PCR method was positive by culture, suggesting that this assay seems to produce no false-negative results in cases of positive blood cultures. Negative blood culture results in the majority of cases, does not exclude sepsis in patients. Negative blood cultures in sepsis may be due to very low number of circulating microbes, uncultivable organisms, fastidious microorganisms or to antibiotic treatment initiated before blood sampling [39]. In HM patients, bacterial translocation may occur from the gastrointestinal tract, oral cavity, or from the outside, which we are not able to isolate using conventional diagnostic methods [40].

The results of nested PCR versus blood culture in the current study are consistent with previous studies [11,20,31]. Samples positive by nested multiplex PCR, but negative by blood culture may raise the suspicion of false positive results, but this seems unlikely, with the use of negative control in each run. All precautions to prevent DNA contamination were taken.

Patients with positive nested PCR and negative blood cultures had high CRP levels ranging from 8.2 to 203mg/L rendering the positive findings of PCR clinically relevant. The absence of a reliable diagnostic gold standard is a common limitation for assessment of new molecular techniques [41]. Therefore, a clinical assessment made by a panel of experts based on the whole range of information available for each patient is a suitable reference standard for diagnostic evaluations of PCR [42]. Other limitations of the current study are that it was carried on one center, and we didn’t use probes for definite identification of microbial species due to lack of financial support.

The primary advantage of a PCR-based assay is obtaining results within short time. Microbial DNA isolation took around 45 minutes using the protocol provided by QIAamp DNA blood Mini Kit. The 1st PCR run took a total time of 3 hours (including gel electrophoresis) while the 2nd run took around 1 hour and 30 minutes. Total time was estimated to be 6 hours compared to at least 72 hours by culture.

This may allow the physician to narrow antimicrobial coverage (bacterial or fungal) early in the course of treatment, thus avoiding the toxicity and costs associated with the use of broad and empirical antimicrobial therapy. PCR can also identify an infection that was not being adequately treated [43,44].

We hope to explore other molecular methods that could speed up the diagnosis of blood stream infections with reasonable cost.

Conclusion

Nested multiplex real time PCR is a promising tool to achieve rapid diagnosis in cancer patients with clinical suspicion of bloodstream infections. Its use in combination with classic methods for early identification of the pathogen could affect the antimicrobial treatment and, therefore, the patient management and outcome.
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