



Original Research Article

Rapid Diagnosis of Invasive Fungal Infections

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ABSTRACT

Keywords

Invasive fungal infection, PCR, ELISA, Diagnosis, Fungi

The incidence of invasive fungal infections (IFIs) has increased considerably in recent years. Invasive fungal infections remain an important cause of morbidity and mortality. New diagnostic approaches have been developed based on non-culture based methods which may allow early diagnosis and treatment of fungal infections. The aim of this study is how to rapidly diagnose IFIs in critically ill patient, comparing the conventional diagnostic method (blood culture) with the rapid diagnostic methods (ELISA and PCR). The study was conducted on 79 patients (51 males and 28 females) admitted to ICU units in Zagazig University hospital and suspected clinically to suffer from invasive fungal infections. Twenty milliliters of blood was collected from each patient during febrile episode. The blood samples were subjected to culture, ELISA and PCR. Considering the different methods used for diagnosis of IFIs the present study shows that 19 cases (24.1%) were +ve blood culture. This number increases to 41 (51.9%) by the use of Ag detection by ELISA and further increase to 47 (59.5%) cases out of the examined 79 patients was by panfungal PCR. PCR is superior to conventional methods for the diagnosis of IFIs. In diagnosis of IFI using conventional methods of diagnosis negative result do not rule out infection, so it is essential to use other diagnostic tools. Also, molecular diagnostic tools are promising and display high sensitivity and specificity.

Introduction

The incidence of invasive fungal infections (IFIs) has increased considerably in recent years because of the increasing population with Human immunodeficiency virus (HIV) infection, transplant recipients, cancer patients, and other individuals receiving immuno-suppressive treatments (Badiee *et al.*, 2009).

Despite the availability of new antifungal drugs, the overall survival for immuno-

compromised patients with invasive fungal infections remains too low, with large variations according to underlying disease (Pasqualotto and Denning, 2005). Invasive fungal infections remain an important cause of morbidity and mortality. The most common fungi that cause disease in transplant recipients and other immuno-compromised patients are *Candida* and *Aspergillus* species (Erjavec and Verweij, 2002).

Although conventional diagnostic tests such as histology, microscopy, and culture remain the cornerstone of proving the presence or absence of fungal disease, their yield is low and, therefore, their impact on clinical decisions to treat patients is limited. Furthermore, cultures become positive at a late stage of infection and delayed therapy is associated with a poor outcome (Maschmeyer *et al.*, 2009).

New diagnostic approaches have been developed based on non-culture based methods, which may allow early diagnosis and treatment of fungal infections (Badiee *et al.*, 2009).

Among the most promising diagnostic techniques are the detection of fungal antigens and molecular biological methods such as Polymerase chain reaction (PCR) (Erjavec and Verweij, 2002).

Galactomannan is a cell wall polysaccharide released by *Aspergillus* spp. during fungal growth in tissue. A commercially available sandwich enzyme-linked immunosorbent assay (ELISA) detects galactomannan by use of a rat monoclonal antibody (Pasqualotto and Denning, 2005).

Circulating galactomannan could be detected in the serum before diagnosis was made by clinical examination and radiology in approximately 65% of patients and at the same time as conventional diagnosis in 10% of patients. However, in approximately 25% of patients circulating antigen was detected after diagnosis was made (Erjavec and Verweij, 2002). 1,3-Beta-D-glucan is also a component of the cell wall. However, unlike galactomannan, which is *Aspergillus* specific, the glucan is present in many fungal species and therefore it should be considered as a pan-fungal detection method and a good indicator of systemic fungal

infection, if detectable in blood or other normally sterile body fluids (Cuenca-Estrella *et al.*, 2008).

Several techniques have been commercialized for serum determination of this compound, although the most widely used is Fungitell, which is also an ELISA technique (Cuenca-Estrella *et al.*, 2008).

Mannan is a cell wall surface carbohydrate that circulates during infection with *Candida* spp., and studies have shown a correlation between detectable mannanemia and tissue invasive in patients with candidaemia (Pasqualotto and Denning, 2005).

The detection of microbial Deoxyribonucleic acid (DNA) by PCR is without doubt one of the most powerful tools for the early diagnosis and identification of human pathogens.

The amplification of gene sequences unique to fungi may allow for early diagnosis of invasive fungal infections and subsequent treatment (Ribeiro *et al.*, 2006).

Although antigen detection has shown variable performance in large clinical studies revealing both benefits and limitations, the availability of standardized protocols and quality assurance provided by commercial kits have allowed their inclusion in the diagnostic criteria for IFD established by the European Organization for Research and Therapy of Cancer and Mycoses Study Group (EORTC/ MSG) Consensus Group. The potential value of nucleic acid-based approaches to the detection and identification of fungal pathogens in immunocompromised patients is indisputable, as shown by a number of studies. However, the lack of standardization and validation across a wide spectrum of diagnostic laboratories has

prevented the inclusion of these tests in the diagnostic criteria of IFD proposed by the EORTC/MSG Consensus Group (Bašková and Buchta, 2012).

This study was designed to assess the abilities of non-culture based methods (Enzyme linked immunosorbent assay for detection of fungal antigen and PCR using panfungal primer for detection of fungi in blood) in the diagnosis invasive fungal infections and to compare their results with those obtained by conventional methods (Blood culture) in order to devise a method for diagnosis invasive fungal infections which is more rapid and accurate.

Materials and Methods

The cross-sectional study was conducted at Zagazig University Hospitals, Egypt on 79 patients (51 males and 28 females) admitted to ICU units and suspected clinically to suffer from invasive fungal infections. An informed consent was obtained from patients before enrollment. The study gained approval of the institutional committee of Zagazig University.

Cases were defined as possible invasive fungal infection according to modification of EORTC/MSG Consensus Group definitions (De Pauw *et al.*, 2008).

Inclusion criteria for patients were:

1. Neutropenia ($<1.0 \times 10^9$ /liter) or prolonged use of corticosteroid or other immunosuppressant.
2. Fever (body temperature >38.5) for > 96 hours refractory to appropriate broad-spectrum antibacterial treatment.
3. Not on regime for antifungal therapy.

Twenty milliliters of blood was collected

during febrile episode under complete aseptic conditions using a sterile syringe and needle, after preparing the patient skin with alcohol soaked pad.

The blood sample was divided into three parts that was stored as follows:

-First part consists of 16 ml of blood was immediately inoculated bedside patient into two blood culture bottles.

-Second part consists of 2 ml of blood transferred to sterile tube and left to clot then centrifuged; the serum was stored in a sterile plastic aliquot at -20°C till the time of serological testing.

-Third part consists of another 2 ml of blood was put in tube containing EDTA that was stored at -20°C for doing PCR.

These samples were used for:

Culture and identification of yielding fungi

Blood culture was done on conventional blood culture bottles. Subculture was done on SDA with chloramphenicol at 25°C , and SABHI Agar Base with chloramphenicol at 37°C . Culture plates were incubated for at least 4 weeks. Plates were evaluated daily for the first seven days and at least twice per week thereafter.

Yeasts and yeast like colonies were identified by colony morphology, pattern of budding, presence of capsules, germ tube formation, and subculture on Brilliance Candida agar medium (Oxoid, England). Filamentous fungi were identified both macroscopically and microscopically by morphology, color, septation of hyphae, pigmentation, pattern of branching, and fruiting bodies.

Serological test:

Mannan antigen was measured in serum using a commercial sandwich immunoassay, Platelia *Candida* Ag (BioRad, Marnes La Coquette, France). The test was performed according to the instructions of the manufacturer.

Galactomannan antigen was measured in serum using a commercial sandwich immunoassay, Platelia *Asprigellus* Ag (BioRad, Marnes La Coquette, France). The test was performed according to the instructions of the manufacturer.

PCR: using panfungal primer for detection of fungi in blood.

DNA extraction (Springer *et al.*, 2012): Extraction of fungal DNA from EDTA whole blood was done using A QIAamp UCP (for ultraclean production) Pure Pathogen Blood Kit (Qiagen, Germany) according to the manufacturer's instructions.

The use of a mechanical disruption using Pathogen Lysis Tubes with large (L) beads to ensure maximal lysis efficiency of the rigid fungal cell wall was recommended QIAGEN.

Blood sample used as negative control was obtained from a healthy volunteer. While positive control used to verify extraction efficiency was prepared by spiking blood sample obtained from healthy volunteer with approximately 150 CFU of *C. albicans* in a volume of 500 ml.

Amplification (Karakousis *et al.*, 2006 & Ribeiro *et al.*, 2006):

Amplification of fungal DNA was performed using universal primers targeting a highly conserved region of the fungal 18S small-subunit rRNA multicopy gene to

generate a PCR product from fungal DNA of 500 bp. The used primers were 18S-forward primer 5'-ATTGGAGGGCAAGTCTGGTG- 3' and 18S-reverse primer 5'-CCGATCCCTAGTCGGCAT-3' (Biolegio, Netherlands).

Samples were processed at the Medical Mycology in Medical Microbiology and Immunology Department, Faculty of Medicine, Zagazig University, Egypt.

All media included were prepared according to the manufacturer's instructions.

Statistical analysis

All data were collected, tabulated and statistically analyzed using SPSS 18.0 for windows (SPSS Inc., Chicago, IL, USA) & MedCalc 13 for windows (MedCalc Software bvba, Ostend, Belgium). Quantitative data were expressed as the mean \pm SD & median (range), and qualitative data were expressed as an absolute frequencies "number" & relative frequencies (percentage). Percent of categorical variables were compared using Chi-square test.

Agreement between the three methods was analyzed using and Kappa statistic, $\alpha = 0.05$. Agreement was obtained the Kappa statistic was significant.

The degree of agreement was very good if the kappa value was 0.81–1.00, good if it was 0.61–0.8, moderate if it was 0.41–0.6, fair if it was 0.2–0.4 and poor if it was < 0.2 . All tests were two sided. $p < 0.05$ was considered statistically significant (S), $p < 0.01$ was considered highly statistically significant (HS), and $p \geq 0.05$ was considered non statistically significant (NS).

Results and Discussion

In this study 79 patients were included, they were 51 males (64.6%) and 28 females (35.4%). Their age ranged from 22 to 69 years with a mean age of 48.1 ± 11.8 y, with the highest age group in the age >52 years old.

For the host factors distribution according to (EORTC/MSG), 59.5% of the studied patients were under corticosteroid therapy, 29.1% were neutropenic, while 11.4% of cases were on immune-suppressive therapy. Each patient in the study had one or more risk factors of the following: solid organ cancer (31.7%), hematological malignancy (25.3%), mechanical ventilation (18.9%), autoimmune disease (13.9%), COPD (13.9%), abdominal surgery (11.4%), diabetes mellitus (11.4%), total parenteral nutrition (10.9%), central venous catheter (7.6%), organ transplantation (5.1%) and renal failure with hemodialysis (3%) with statistically significant difference ($P < 0.001$) (Figure 1).

Using conventional methods presented by blood culture 19 samples were positive, of which yeasts accounted for 78.9% and moulds accounted for 21.1%. Yeasts isolated were caused by different *Candida* species, the majority of *Candida* infections were caused by *C. albicans* accounting for 53.3%, whereas non *C. albicans* species accounted for 46.7% of *Candida* infections, those include *C. glabrata* 26.7% and *C. krusi* 20.0% of yeast infections. The majority of isolated moulds were caused by *Aspergillus* species, of which *A. fumigatus* was responsible for 50.0%, while *A. niger* accounted for 25% of mould infections. *Fusarium* species was isolated from one blood culture accounting for 25% of mould infections (Table 1).

Using non conventional methods, 37.9% of patients enrolled were positive by Platelia™ *Candida* Ag detection test. 13.9% were positive by Platelia™ *Aspergillus* Ag detection test and 59.5% were positive by Panfungal PCR, with statistically significant difference ($P < 0.05$) (Table 1 and Figure 2).

Comparing the results of different methods used for diagnosis of IFIs, 19 cases (24.1%) were +ve blood culture. This number increases to 41 (51.9%) by the use of Ag detection test and further increase to 47 (59.5%) cases out of the examined 79 patients was by panfungal PCR.

Accordingly, ELISA can detect all the blood culture positive cases and 22 of the blood culture negative cases. The agreement between the results of blood culture and Ag detection test of fungal antigen in serum by ELISA was moderate ($\kappa = 0.43$). PCR can detect all the blood culture positive cases and 28 of the blood culture negative cases. The agreement between the results of blood culture and panfungal PCR was fair ($\kappa = 0.34$). At the same time, PCR can detect all the ELISA positive cases and six of the ELISA negative cases. The agreement between the results of Ag detection test of fungal antigen in serum by ELISA and panfungal PCR was very good ($\kappa = 0.89$) (Tables 2 and Figure 3).

Concerning time taken by each method to diagnose IFIs, blood culture method took the most time (up to two weeks) followed by Ag detection (3–4 hours), while PCR took the least time (1–2 hours).

According to EORTC/MSG, 24% of studied patients were proven invasive fungal infection, 27.8 % were probable invasive fungal infection and 48.1% were possible

invasive fungal infection (among them six cases were PCR positive) (Table 3).

Invasive fungal infections constitute a serious threat to an ever-growing population of immunocompromised individuals and other individuals at risk. Traditional diagnostic methods, such as histopathology and culture, which are still considered the gold standards, have low sensitivity, which underscores the need for the development of new means of detecting fungal infectious agents (Arvanitis *et al.*, 2014).

Establishing a definite diagnosis of IFI in immunocompromised patients is particularly challenging and time consuming, but delayed initiation of antifungal treatment increases mortality. In the last few years, diagnostic procedures have been vastly improved, particularly noteworthy is the introduction of newer imaging techniques and non-culture methods, including antigen-based assays, metabolite detection and molecular detection of fungal DNA from body fluid samples (Ruhnke *et al.*, 2012).

In the present study, the highest age group was in the age >52 years old. This is in consistent with the work of Meersseman (2004) & Montagna *et al.* (2013) where most of cases of invasive fungal infection tend to occur in high age (older than 60 years), and this can be attributed to the increased incidence of invasive mycoses with bipolarity of age in association with diminished immunity and body resistant. On the contrary, Soliman *et al.* (2012) detected most of invasive fungal infection cases within younger age group, as this study was conducted on patient with hematological malignancy which show higher incidence in this group of age.

As regarding host factors distribution, it was found that 59.5% of the studied patients were under corticosteroid therapy, 29.1%

were neutropenic, while 11.4% of cases were on immune-suppressive therapy. This is in agreement with Meersseman *et al.* (2004) who found that the highest percentage of studied patients was under corticosteroid therapy, followed by patients who were neutropenic, then those who were on immune-suppressive therapy. Similarly, in the study of Slavin *et al.* (2010) the highest percentage of studied patients were under corticosteroid therapy while cases that were on immune-suppressive therapy and neutropenic cases nearly were same percentage.

High incidence of IFI with corticosteroids is due to suppression of the immune system, especially the cellular component (Sipsas and Kontoyiannis, 2012). Meersseman *et al.* (2004) explained that Corticosteroids substantially impair macrophage killing of *Aspergillus* spores and mononuclear cell killing of *Aspergillus* hyphae, also threshold steroid level varies according to the type of patients, and emphasized that in the setting of underlying lung disease there is a risk factor for IA at much lower doses.

Distribution of risk factors among the studied patients with clinical suspicion of IFIs revealed a high association of IFIs with solid organ cancer (31.7%) and hematological malignancy (25.3%). This result is consistent with Slavin *et al.* (2010) who found that the highest percent of studied patients had solid organ cancer followed by those who had hematological malignancy. Also, in the study of Montagna *et al.* (2013), percentage of cases with solid organ cancer was higher than cases of hematological malignancy.

Yeast was found to account for 78.9% and mould for 21.1% of all infections and this is in consistent with Montagna *et al.* (2013), where yeast accounted for 87.6% and moulds accounted for 12.4% of all infections. These results can be explained as

follows: Unlike invasive candidiasis, which can affect relatively noncompromised patients, invasive disease caused by *Aspergillus* spp., moulds generally involves severely immunocompromised patients. Also, most of *Candida* infections are endogenous while *Aspergillus* infection is mainly exogenous. However, George (2011) detected higher incidence of mould than yeast among all infections.

Yeast was isolated from 19% of studied patients. These results are in agreement with that of Yang *et al.* (2006) who found positive blood culture for *Candida* in 13.5% of studied patients. However a lower rate of detection (7.5%) was reported by Abelson *et al.* (2005).

The majority of *Candida* infections 53.3% were caused by *Candida albicans*, whereas 46.7% were caused by non *C. albicans* species. This result was consistent with Tortorano *et al.* (2012) study, which showed that *Candida albicans* accounted for 60% of *Candida* infections.

On the contrary, Montagna *et al.* (2013) found that the majority of candida infections 59.8% were caused by non *C. albicans* species and *Candida albicans*, accounted for 40.2% of yeast infection, and attributed the reason for this change in the pattern of *Candida* spp. distribution to some predisposing factors, such as indwelling catheters and parenteral nutrition for *C. parapsilosis*, cancer and neutropenia for *C. tropicalis*, and previous exposure to azoles for *C. krusei* and *C. glabrata*. However, in nonneutropenic patients, risk factors for *C. krusei* nosocomial candidemia include recent gastrointestinal surgery and exposure to fluconazole (Playford *et al.*, 2008).

Isolated non *C. albicans* species included: *C. glabrata* which formed 26.7% of yeast

infections, and *C. krusei* which formed 20.0%, of yeast infections. In a study done by Tortorano *et al.* (2012), it was found that the incidence of *C. glabrata* was 13.4% and *C. krusei* was 1.8%. Similarly, in Montagna *et al.* (2013) study *C. glabrata* formed 10% of yeast infection.

The clinical significance of the isolation of non *albicans Candida* spp. is the increased likelihood of resistance to fluconazole and other azoles. In particular, of the commonly isolated non *albicans Candida* spp, fluconazole resistance was noted in 16% of *C. glabrata*, and 78% of *C. krusei* (Hachem *et al.*, 2008).

Furthermore, it was found that *C. krusei* was the fifth most common *Candida* spp. isolated and accounted for 2.5% in a global antifungal surveillance program (Pfaller *et al.*, 2008). *C. krusei*, intrinsically resistant to fluconazole, has often been reported in patients with hematologic malignancies with prior fluconazole and antifungal exposure and is associated with the highest mortality of all *Candida* spp. (Horn *et al.*, 2009).

As regarding moulds which were isolated from 5.1% of studied patients, the majority of the mould infections (75%) were caused by *Aspergillus* spp, of which *A. fumigatus* was responsible for 50% and *A. niger* was responsible for 25% of mould infections. While, *Fusarium* spp. accounted for 25% of mould infections. However, Girmenia *et al.* (2001) reported that only 10% of blood cultures were positive in patients with Invasive aspergillosis. These findings were consistent with (Ulrike and Lass-Flörl, 2011) who mentioned that the most common species causing aspergillosis is *A. fumigatus*, also Jane *et al.* (2011) who found that *A. fumigatus* causes 44% of invasive aspergillosis cases. *A. fumigatus* is more thermotolerant than other disease-causing

species, growing well at 37°C, and several studies suggested that the radial growth and germination rate of aspergilli at 37°C correlate with pathogenicity. Moreover, being resistant to oxidative stress; *A. fumigatus* detoxifies oxidative threats via glutathione synthesis and oxidoreductase activity (Dagenais and Keller, 2009).

Furthermore, Ulrike and Lass-Flörl (2011) found that the most common species causing aspergillosis is *A. fumigatus*, accounting for approximately 90% of aspergillus infections. Depending on regional distinctions *A. flavus*, *A. nidulans*, *A. terreus* and *A. niger* are frequently reported as well, and there is evidence that these non-*fumigatus* pathogens are increasingly common etiologic agents, in the current study other *Aspergillus* spp. also recovered from patient samples, where *A. niger* accounted for 25% of mould infections.

In our study, another filamentous fungus which is *Fusarium* was found to be responsible for 25% of mould infections. Fusariosis is a life-threatening and increasingly important mycosis in immunocompromised hosts. Risk factors for such infections are skin lesions, burns, use of corticosteroids, prolonged neutropenia and hematological malignancy (Ulrike and Lass-Flörl, 2011). Other studies have reported 3-month mortality rates of 80% in hematopoietic cell transplantation from infections due to *Fusarium* species (Jane *et al.*, 2011). Nucci and Anaissie (2007), reported that blood cultures are frequently positive in fusariosis and yielded the organism in 41% of the cases. This is possibly due to the fact that *Fusarium* species produce yeast like structures (adventitious sporulation) that facilitate their dissemination and growth in the blood.

Isolation of etiologic fungal agent by culturing

different clinical specimens is considered one of the methods used for the diagnosis of IFI, provided that the samples are from sterile sites such as blood, tissue or cerebrospinal fluid, in which fungal infection can be documented, although, the sensitivity of blood culture for the diagnosis of fungal infection is controversial. Multiple or repeated blood cultures should be performed to increase the likelihood of detecting candidemia, and filamentous fungi are rarely isolated from blood. So far, culture can be useful to determine the sensitivity of the isolated fungi to antifungal agents and identify resistant species, and are thus needed to optimize patient management. As a result, given the limitations for diagnosis of IFI, negative result on direct or pathologic smears and cultures do not rule out infection, so it is essential to use other methods (Badiee and Hashemizadeh, 2014).

In order to overcome the limitations of conventional diagnostic tests, several studies have focused on the development of non-culture based methods with superior sensitivity and quicker turnaround time, such as the detection of fungal components or metabolites, as an alternative approach for the early detection and identification of the causative fungal species (Yeo and Wong, 2002).

Serological tests for detection of fungal antibodies have been the subject of much study but can be difficult to interpret. For example, circulating antibodies to *Candida* spp. may occur in normal individuals as a result of commensal colonization of mucosal surfaces, antibody production in the immuno-compromised patient population varies according to immune status and false negative results occur in immunocompromised patients who produce low or undetectable levels of antibodies. However, the antigen detection tests are

more significant for diagnosis because they detect active infection. Antigen is generally detected in serum, yet, it can also be detected in other body fluids, such as urine (Sendid *et al.*, 2002).

Using Platelia™ *Candida* Ag detection test, 37.9% patients enrolled were positive. Accordingly Ag detection test detected all of the blood culture positive cases and 25% of the blood culture negative cases.

These results are in agreement with Montagna *et al.* (2013). Unlike Meersseman *et al.* (2004) who reported a rate of 40% for antigen detection among patients.

Interestingly, the possible cause of the variability in sensitivity of the serum GM assay is the level and nature of immune suppression of the patient. Classically, Patients with Low neutrophils levels are more likely to have higher serum GM levels than patients with higher neutrophil levels, as when the assay is applied to patients with hematological malignancy or having undergone bone marrow or stem cell transplantation. Also, serum GM assay has been shown to be modestly sensitive in COPD patients with IA and poorly sensitive in solid organ transplantation. The lower incidence of neutropenia in these two patient groups may well account in part for the reduced sensitivity of the serum GM assay. Also, this result could be logical in patients receiving antifungal therapy where antifungal treatment inhibits growth of *Aspergillus* renders nonviable reducing the load of fungal cells able to shed the GM antigen (Barton, 2013).

The detection of non-cultivable/nonviable cells and circulating free fungal DNA by PCR has been described as an important tool in the early diagnosis of IFI, unlike in culture which needs viable cells. Therefore, diagnostic assays based on in vitro

amplification and detection of fungal DNA has been developed. PCR methods are particularly promising because of their high specificity and sensitivity (Chen *et al.*, 2001).

About 59.5% of enrolled patients were positive by panfungal PCR. However, 24.1% of cases were +ve blood culture. Increased to 51.9% of cases by the use of Ag detection test and further increased to 59.5% of examined cases by panfungal PCR, with statistically significant difference ($P < 0.05$)

These results are coincident with the work of El-Sayed *et al.* (2012) who detected 57.7% of cases positive by panfungal PCR, consequently 88% of the positive patients and 50% of all were missed by blood culture. Also, another study stated that 64.5% were positive by both culture and PCR, of these samples 19.4% showed no growth of fungi but were positive by PCR (Gurtner *et al.*, 2007). On the other hand, results of Badiie *et al.* (2009) study revealed that 10.6% of patients had positive cultures for fungal infections whereas 17.7% were positive for fungal infection by panfungal PCR. Ribeiro *et al.*, (2006) study results revealed that 35% of enrolled patients were positive by panfungal PCR.

Khot *et al.* (2009) mentioned that the ability of panfungal PCR assays to detect a wide spectrum of fungi is clearly an advantage. Conversely, amplification of contaminating fungal nucleic acids present in the environment or laboratory can be a drawback. In general, panfungal PCR assays could be useful when applied to a clinical sample as part of a larger array of assays. For example, panfungal PCR assays could detect new pathogens previously unreported, and identify etiological agents in mixed infections, especially when used with other genus-specific assays.

Blood is the most sterile of the clinical samples used for diagnosis by PCR; therefore, it is more useful for applying panfungal PCR assays because a PCR positive provides more convincing evidence of fungal disease. On the other hand, use of panfungal PCR on nonsterile clinical samples, especially BAL fluid, for the diagnosis of IA may result in suboptimal results – low specificity due to cross-reactivity with commensal fungi such as *Candida* spp. present in the oral cavity, and low sensitivity because these commensal fungi may compete with the pathogen for amplification (Khot and Fredricks, 2009).

The diagnosis of patients with IFIs in the present study revealed a very good agreement between Ag detection test of fungal antigen in serum by enzyme immunoassay and panfungal PCR. However, there is moderate agreement between the results of blood culture and Ag Detection Test. Additionally there is a fair agreement between the results of blood culture and panfungal PCR. In accordance with our results El masry *et al.* (2005) also detected a very good agreement (94.4%) between the two non-culture based methods (PCR and ELISA).

Studies by Florent *et al.* (2006) and White *et al.* (2006) that compared GM antigen and PCR assays found that when used together the sensitivity of diagnosis can be increased. Accordingly, they concluded that complementary use of these tests may reduce dependence on invasive diagnostic procedures and limit the need for empirical antifungal therapy.

El-Sayed *et al.* (2012) detected poor agreement between the results of blood culture and panfungal PCR ($\kappa = 0.10$). They conferred the discrepancies in the

results of culture and PCR to that the detection of fungemia by means of fungal blood culture is notoriously difficult and IFIs are diagnosed better by molecular assay as naked DNA can be detected by PCR due to the presence of dead and degrading fungi within circulating phagocytes (Ahmaad *et al.*, 2002). Also, the time factor could offer another explanation for the observed differences in the results of blood culture and PCR. According to Estrella *et al.* (2009), fungal DNA was detected by PCR much earlier than GM in patients with IFI.

Generally, the diagnosis of infection based on molecular and serologic techniques can provide powerful tools for the early and rapid diagnosis of IFI. As the fungi are common in the environment and opportunistic fungi in immunocompromised patients can cause high morbidity and mortality, the interpretation of positive or negative results with different laboratory methods is difficult for clinicians, so more than one method should be used for early diagnosis (Badiee and Hashemizadeh, 2014).

Concerning time taken by each method to diagnose IFIs, blood culture method took the most time (up to 2 weeks) while PCR took the least time (1–2 hours). Inning *et al.* (2007) stated that although blood culture technique is still used for routine diagnosis of IFIs, blood cultures are slow and lack the desired sensitivity and specificity.

Invasive fungal infections in our study were defined using EORTC/MSG Consensus Group criteria (De Pauw *et al.*, 2008), and according to these criteria we found that 24% of cases were classified as proven IFI, 27.8 % were probable IFI and 48.1% were possible invasive fungal infection (among them 6 cases were PCR positive).

Table.1 Mycological findings of patients with clinical suspicion of invasive fungal infection (N=79)

Mycological findings	Patients with clinical suspicion of invasive fungal infection (N=79)	
	No.	%
Blood culture		
Negative	(60)	(75.9%)
Positive	(19)	(24.1%)
Yeast*	15	19%
Mould**	4	5.1%
ELISA		
Negative	(38)	(48.1%)
Positive	(41)	(51.9%)
Candida Ag	30	38%
Aspergillus Ag	11	13.9%
Panfungal-PCR		
Negative	32	40.5%
Positive	47	59.5%

*Yeast species include *C. albicans* (No.8), *C. glabrata* (No.4) and *C. krusi* (No.3)

**Mould species include *A. fumigates* (No.2), *A. Niger* (No.1) and *Fusarium* (No.1)

Table.2 Agreement between Blood culture, ELISA and PCR used for diagnosis of IFIs

Test	Agreement %	Kappa*	P value
Blood culture versus Ag detection test	69.6	0.43	0.00
Blood culture versus panfungal PCR	63.1	.34	0.00
Ag detection versus panfungal PCR	92.4	.89	0.00

*value of K: Strength of agreement <0.2: poor 0.2-0.4: fair 0.41-0.6: moderate 0.61-0.8: good 0.81-1.00: very good

Table.3 Classification of cases according to EORTC/MSG criteria

Class	Criteria	Patients with clinical suspicion fungal infection (N=79)	
		No.	%
Proven			
Fungemia proven by blood cultures that gave fungal growth		(19)	(24%)
Probable			
Host factor: (Neutropenia or prolonged use of corticosteroid or other immunosuppressant) & Clinical criteria: (fever, cough, dyspnea, ronchi, hemoptysis,) & Mycological criterion: Indirect tests (GM & Manan Ag detection in serum)		(22)	(27.8%)
Possible			
Host factor: (Neutropenia or prolonged use of corticosteroid or other immunosuppressant) & Clinical criteria: (fever, cough, dyspnea, ronchi, hemoptysis,)		(38)	(48.1)

Figure.1 Distribution of risk factors of patients with clinical suspicion of invasive fungal infection (N=79)

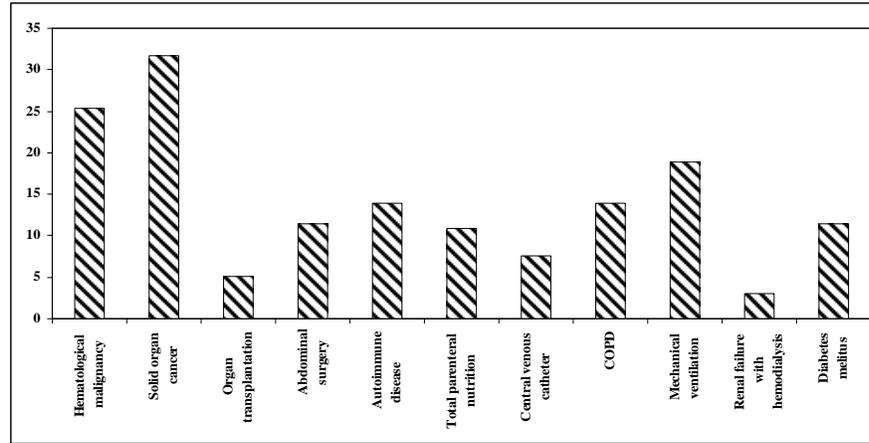
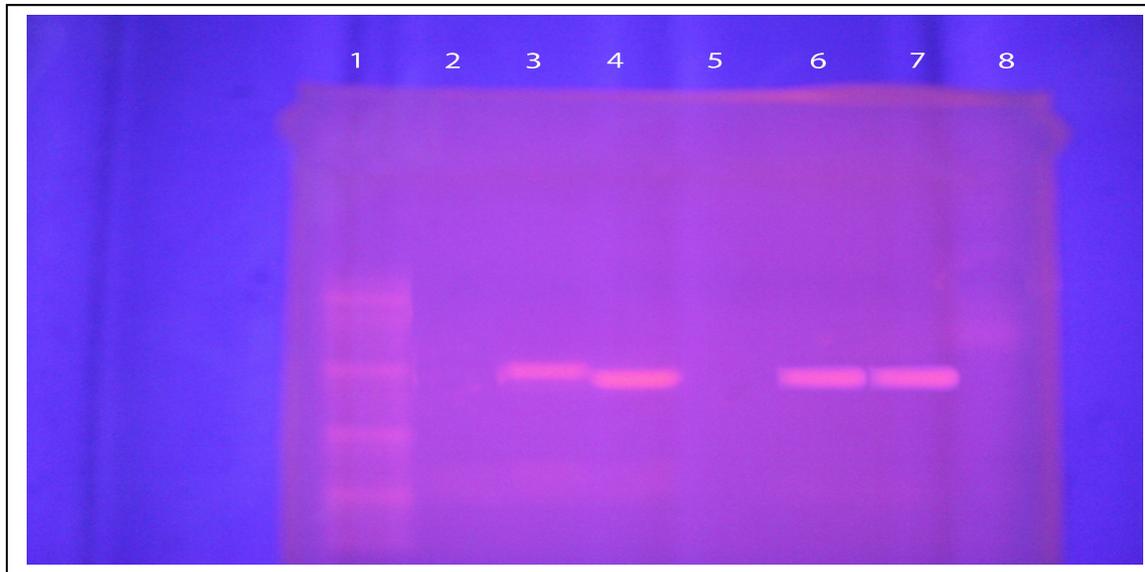


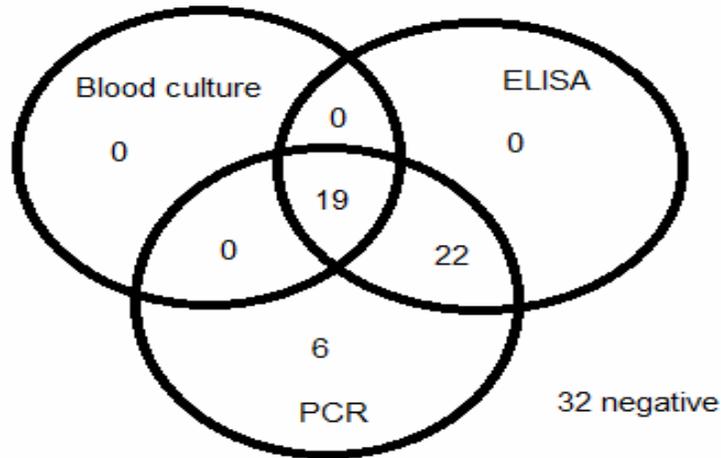
Figure.2 PCR using panfungal primer



Lane 1: Molecular size marker (50-1500bp)
Lane 3: Positive control
Lane 5: Negative case
Lane 7: Positive case

Lane 2: Negative control
Lane 4: Positive case
Lane 6: Positive case
Lane 8: Negative case

Figure.3 Venn diagram showing number of positive results in each combination of blood culture, ELISA and PCR (n. 79)



In the current study, PCR assay for the detection of fungal nucleic acids appear to be promising diagnostic approach. Meanwhile, PCR is not considered within the EORTC/MSG criteria. Landinger *et al.* (2010) had focused on the correlation between PCR positive patients and the presence of proven, probable, and possible criteria of IFI according to the EORTC-MSG criteria; 150 febrile neutropenic pediatric patients with high risk of IFI were investigated by panfungal PCR in his study, all proven, probable IFI, and one case with possible IFI were PCR positive.

Many studies show a tendency of PCR methods to detect fungal DNA in patients at high risk of IFI with negative blood culture. In some cases of discordant results between blood cultures and PCR, fungal species were isolated from the same population of patients, from another sterile site, thus indicating that the PCR result was in fact positive and proving the superiority of the PCR assay over the traditional diagnostic test (Arvanitis *et al.*, 2014).

It was concluded that conventional diagnostic tests such as histology, microscopy, and culture remain the cornerstone of proving

the presence of fungal disease, while rapid diagnostic techniques offer a powerful tool for the early diagnosis and identification of fungal pathogens.

Concerning the time consumed to diagnose IFIs, blood culture method took the most time while PCR took the least time. Furthermore, PCR provides accurate and reproducible method, and it is clearly advantageous regarding speed, handling and number of samples that can be analyzed per run and fulfills the need for rapid identification.

The recommendations includes in diagnosis of IFI using conventional methods of diagnosis a negative result does not rule out infection, so it is essential to use other diagnostic tools. Clinicians must be aware of and familiar with how best to utilize the newer diagnostics such as Ag detection test of fungal antigen and PCR assays, in conjunction with older diagnostic techniques, to improve the care of patients with IFI. Also, Molecular diagnostic tools are promising and display high sensitivity and specificity. It is important to consider standardization of fungal PCR so it can be included as mandatory in the

recommendations. Lastly, Future efforts should focus on further improvements of rapid diagnostic techniques, which would allow for the timely application of antifungal therapy and would reduce the use of treatments in inappropriate settings.

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