Original article

Real-Time PCR in the early detection of invasive fungal infection in immunodeficient infants and children

Background: *Crucial to the diagnosis and effective therapy of invasive fungal infection (IFI) in the immunodeficient is the early identification of the causative agent especially in patients who lack clinical evidence of the disease. The standard methods for the detection of fungi in clinical specimens are direct microscopy and mycological culture. Microscopy often lacks a satisfactory sensitivity, whereas diagnosis by mycological culture often requires a long growth period. Studies have demonstrated the feasibility of detecting molds and yeast in a single reaction using the universal fungal primer.* **Objective:** *Evaluation of the role of real-time PCR in the early detection of fungal infection in immunodeficient patients with suspected IFI, who lack clinical evidence of the disease.* **Methods:** *This study included 30 immunodeficiency patients suspected of having IFI; 9 with primary and 21 with secondary immunodeficiency. All patients had at least one host factor, but no clinical criteria according to the EORTC-MSG definition of IFI. Twenty seven had fever and 3 had bronchopneumonia, both not responding to broad spectrum antibiotics for 96 hrs. or more. Blood samples were cultured for fungi and were analyzed with real-time PCR using universal fungal primers. For positive samples of fungal infection, aspergillus-specific primers were used for detection of aspergillus.* **Results:** *Seventeen patients (56.7%) proved to have IFI. Blood culture detected Candida in 2 patients only, while PCR detected Candida in another 9 and Aspergillus in 6, thus 15/17 patients with IFI (88%) were missed by blood culture. Blood culture for IFI diagnosis had a very low sensitivity (12%) but had a 100% specificity and positive predictive value. The results PCR did not vary with gender, degree of fever, immunodeficiency type, clinical presentation or current intake of antifungal treatment. Patients with proven IFI showed significantly increased CRP levels as compared to those without infection.* **Conclusion:** *Real-time PCR proved superior to culture in early diagnosis of IFI in patients with immunodeficiency before the appearance of the characteristic clinical and imaging signs. Reliance on blood culture alone at that stage would result in missing most of the positive cases with consequent delay in the initiation of specific treatment.*

Keywords: Invasive fungal infection, immunodeficiency, blood culture, real-time PCR, candida, aspergillus.

**Zeinab A. El-Sayed,
Zeinab E. Hasan,
Rasha A.R. Nasr***

Pediatrics, Medical Microbiology and Immunology *Departments, Ain Shams University, Cairo, Egypt.*

Correspondence:
Zeinab Ebraheem Hasan. Department of Pediatrics, Faculty of Medicine, Ain Shams University, Abbasia, Cairo, Egypt
E-mail: *zeinabeh2002@ yahoo.com*

INTRODUCTION

Invasive fungal infections (IFI) in children appear to have increased over the past two decades. Among immunocompromised children, the impact of IFI can be devastating, with a high rate of mortality and morbidity. Timely diagnosis and initiation of appropriate antifungal therapy is imperative for improving outcomes.[1]

Although conventional diagnostic tests such as histology, microscopy, and culture remain the cornerstone of proving fungal disease, their yield is low and, therefore, their impact on clinical decisions to treat patients is limited. Invasive procedures such as biopsy of the infected site may be precluded due to the presence of severe thrombocytopenia. Furthermore, cultures become positive at a late stage of infection and delayed therapy is associated with a poor outcome.[2,3]

Candida and Aspergillus species are the most commonly isolated organisms.[1] Since non-albicans Candida species and non-fumigatus Aspergillus species are increasing in importance, new diagnostic approaches covering a large number of fungal species are required.[3,4] Several studies have demonstrated the principle feasibility of detecting

molds and yeast DNA in a single reaction using the universal fungal primer.[5,6] The introduction of real-time PCR technology in the detection of fungal infections has increased the realibilty of PCR results compared to results obtained by conventional PCR. The absence of post-PCR processing after amplification has sharply decreased the risk of false positive reactions which might occur during the use of acrylamid or agarose gel electrophoresis. Moreover, real-time PCR can give the results in less than 2 hours, a requirement for clinical decision making.[7]

This study aimed to evaluate the role of real-time PCR in the early diagnosis of IFI in immunocompromised patients who lack clinical evidence of the disease.

METHODS

Patients:

This study included 30 patients with immunodeficiency (9 with primary and 21 with secondary immunodeficiency) suspected to have IFI. They were consecutively enrolled from the Pediatric Allergy and Immunology, Hematology and Oncology, and Intensive Care units of Ain Shams University, Children′s Hospital during the period from June 2009 to August 2010. They were 9 females and 21 males with ages ranging from 3 months to 14 years. An informed consent was obtained from the patients' parents or care-givers before enrollment. The study gained the approval of the Ethicas′ Committee of the Department Pediatrics, Ain Shams University.

Twenty seven patients had fever and three had bronchopneumonia, both not responding to broad spectrum antibiotics for a minimum of 96 hours. As per the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) criteria for IFI, all patients had at least one host factor but lacked the characteristic clinical and imaging signs of proven or probable IFI.[8]

A three-page sheet was constructed to collect necessary data through:

Clinical history taking: with special emphasis on details of the original immunodeficiency disease, symptoms suggestive of local and/or systemic infection, type, dose and duration of immunosuppressive therapy including corticosteroids and the antibiotics and/or antifungals received.

Physical examination: including body temperature recording, examination of the oral cavity for ulcers or thrush, looking for nasal ulcers or sinusitis, chest, abdominal and CNS examination, as well as looking for perineal rash.

Imaging studies: including plain radiographs, computed tomography scans, and MRI of suspected sites; done as needed.

Urine analysis and serum biochemical evaluation of liver and kidney functions were done for all patients in addition to Complete Blood Count, CRP titre and Erythrocyte Sedimentation Rate.

Microbiologic assessment:

1-Blood sampling

Five milliliters of venous blood were withdrawn from each patient under complete aseptic condition: 0.5-1.5ml were added to the blood culture bottle for fungal culture and 3 ml were mixed with EDTA in a tube and stored at -20°C until performance of PCR.

2-Blood culture

The blood culture bottles were incubated aerobically at 35°C and were subcultured every 48 hours onto two Sabaroud's dextrose agar and one blood agar (Oxoid, England) plates until growth was detected or the bottle was discarded after two weeks. One Sabaroud's dextrose agar and the blood agar plates were incubated at 35°C for detection of Candida and bacterial isolates. The other Sabaroud's dextrose agar plate was left at room temperature for upto 2 weeks for detection of filamentous fungal growth. All isolates were identified according to Collee et al.[9] and Milne et al.[10] based on colony morphology, microscopic examination of Gram-stained film and their biological activity.

3-Real-time PCR for detection of fungal infection

Total DNA was extracted using MagNA pure compact nucleic acid isolation kit I (Cat.No.03730964001, Roche, Germany) according to manufacturer's protocol. Universal fungal primers as described by White et al. (11) were used for amplification. The sequence of primers is ITS1: 5'-TCCGTAGGTGAACCTGCGG-3' and ITS 4: 5'-TCCTCCGCTTATTGATATG-3'. The ITS region primers make use of conserved regions of the 18S (ITS 1) and the 28S (ITS 4) rRNA genes to amplify the intervening 5.8S gene and the ITS 1 and ITS 2 noncoding regions. For detection of aspergillus in positive samples for fungal infection, the sequence of the primers used were 5'-TTG GTG GAG TGA TTT GTC TGC T-3' and 5'-CTA AGG GCA TCA CAG ACC TG 3', which target Aspergillus-specific sequences of the fungal 18S rRNA gene.[12] Amplification was carried out in the LightCycler 2.0 System (Roche, Germany) using LightCycler-

DNA master SYBR green amplification kit (Cat.No. 2015099, Roche, Germany).

Statistical Methods:
Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS, version15). Data were expressed as mean ±SD (range) or as number (%) of cases. Comparison of proportions and means between both groups was made by using the χ2 (Chi square test) and independent t-test, respectively. The Fisher's exact test was used when applicable. Unpaired (student') t test was used to test the difference between mean values of laboratory parameters, Non parametric data as platelets, age, and weight were presented as median and interquartile range (IQR) and were analyzed using Mann-Whitney test. The level P<0.05 was considered the cut-off value for significance. Kappa test was done to measure the agreement between two observers.

RESULTS
The demographic, clinical and some laboratory data of patients are presented in table (1). Of the studied children, 21 (70%) had secondary immune-deficiency. The etiology of 2^{ry} immunodeficiency was as follows: 8 had acute lymphoblastic leukemia; ALL (38.1%), four had acute myeloblastic leukemia; AML (19.04%), four had neuroblastoma (19.04%), four had lymphoma (19.04%) and one with Wilms tumor (4.7%). The remaining 9 children (30%) with primary immunodeficiency had their diagnoses as follows: three had congenital neutropenia, two had severe combined immunodeficiency (SCID), two with chronic granulomatous disease, one with Wiscott Aldrich syndrome and one with Omenn disease. The 9 patients with 1ry immunodeficiency had one host factor, while all patients with 2ry immunodeficiency had two or more host factors according to the EORTC MSG criteria.[8] All patients with 2ry immunodeficiency were neutropenic and 80.95% of them were treated with antifungal, while only 22.2% of 1^{ry} immunodeficiency were neutopenic and 88.8% of them were treated with antifungal (Figure 1).

Bacteremia was detected in 3 patients: one had *Staphylococcus aureus*, 1 had *Acinetobacter baumannii* and the third had *Pseudomonas*

aeruginosa. These three patients had negative blood cultures for fungi, but by PCR, the first two were positive for Aspergillus and Candida, respectively. Blood culture detected 2 instances of fungal infection, namely Candida, while panfungal PCR detected 17 (57.7%) including those afore-mentioned two instances of Candida. Hence 15 patients (88% of the positive patients and 50% of all) were missed by blood culture. Statistically, there was no agreement between the results of blood culture and panfungal PCR k=0.10 P>0.05 as shown in table (2). Blood culture had a sensitivity of 12% (2/17), specificity of 100% (13/13), PPV of 100% and a NPV of 46% with diagnostic accuracy of 50%.

Of the 17 positive cases by panfungal PCR, 6 patients were positive for Aspergillus by the Aspergillus-specific PCR, hence, 11 patients were considered suffering of candidemia. While 2 of these patients with candidemia were detected by blood culture, the other 9 patients were missed by blood culture. Furthermore, none of the 6 patients with positive PCR for Aspergillus was detected by blood culture.

Of the 9 primary immunodeficiency children, 6 (66.6%) had positive PCR results: 2 for Aspergillus and four were considered Candida. Concerning children with secondary immunodeficiency, 11/21, i.e. 52.3% had positive PCR results: four for Aspergillus and 7 considered were Candida. Therefore, nearly one third of the patients with PCR-proven fungal infection had invasive aspergillosis (IA). IA was seen in 2 SCID patients and 4 neutropenic cancer children while invasive candidosis (IC) was seen in a patient with Wiscott Aldrich, a patient with Omenn's disease, two with congenital neutropenia and 7 neutropenic cancer children.

All the three patients with bronchopneumonia and half of those with fever not responding to antibiotics proved to have IFI. The results of PCR did not differ with age, gender type, degree of fever (< or >39°C), type of immunodeficiency (1^{ry} or 2^{ry}), clinical presentation, current intake of antifungal treatment, weight, duration of current illness, duration of antibiotics, and laboratory parameters. Patients with fungal infection, showed significantly increased CRP level when compared to those without infection P<0.05 as shown in figure (2).

Table 1. Demographic, clinical and laboratory data of patients.

Variable	Results
Age(months)	Median(IQR): 48.00(78.5)
Weight (Kg)	Median(IQR): 13.00(14)
Weight Percentile (%)	Median(IQR): 5.00(7)
Duration of current illness (days)	Mean ±SD: 8.23±4.58
Duration of antibiotic (days)	Mean ±SD: 8.23±4.58
Duration of antifungal (days)	Mean ±SD: 3.43±3.73
White blood cell (x10^3/mm^3)	Mean ±SD: 3.78±5.64
Neutrophil %	Mean ±SD: 33.37±19.63
Absolute neutrophil count	Mean ±SD: 1520±2640
Lymphocytes%	Mean ±SD 51.38±23.07
Absolute lymphocytic count	Mean ±SD: 1820±3250
Hemoglobin (gm/dl)	Mean ±SD: 9.01±2.22
C reactive protein CRP	Mean ±SD: 43.93±36.20
Platelets (x10^3/mm^3)*	Median(IQR): 112(265.75)
Eryhrocyte sedimentation rate ESR	Mean ±SD: 38.67±23.69

IQR: interquartile range

Table 2. Comparison between blood culture and panfungal PCR in detection of fungal infection.

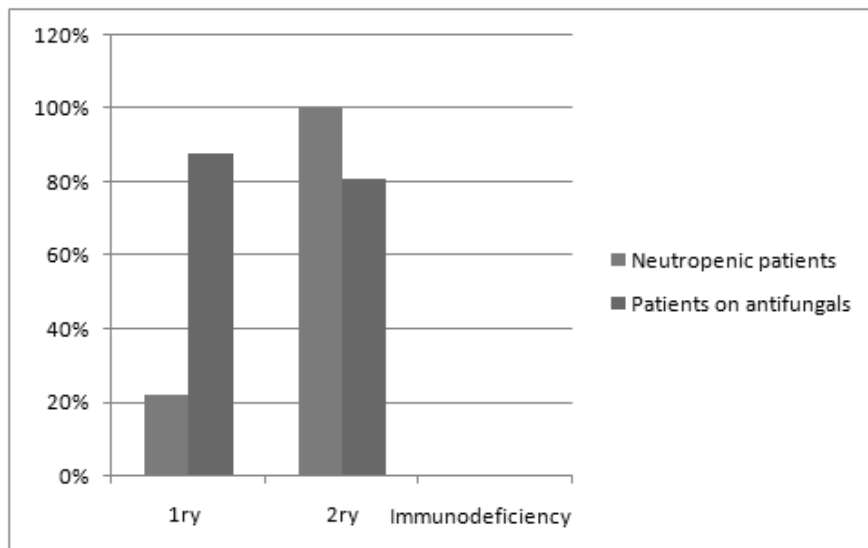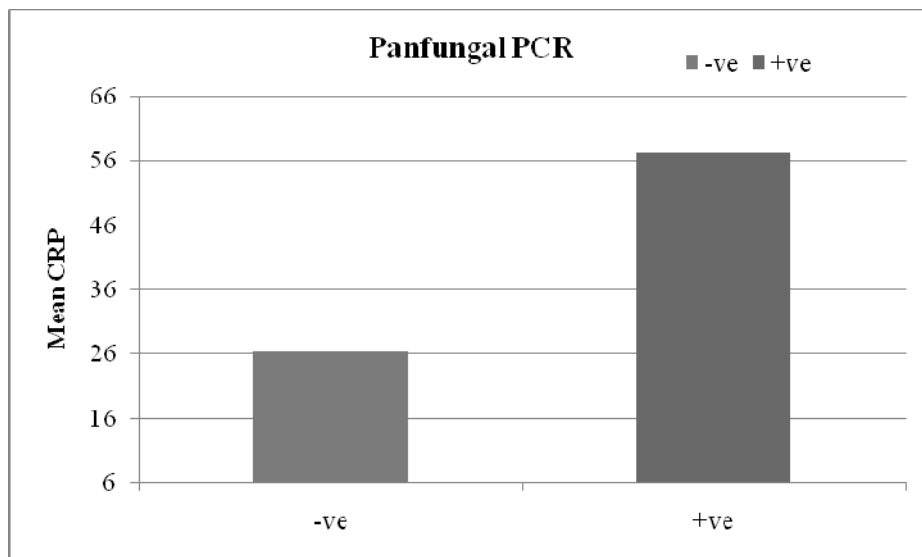| Variable		Panfungal PCR results				k	P	Sig.
		Negative(13)		Positive (17)				
		N	%	N	%			
Fungal blood culture results	Negative (28)	13	43.3%	15	50.0%	0.10	0.20	NS
	Positive (2)	0	0.0%	2	6.7%			

NS: non significant; k: kappa test

Figure 1. Neutropenic patients and patients on antifungal therapy among the immunodeficient children.

P <0.05: significant
Figure 2. Variation of the C reactive protein (CRP) levels with the results of panfungal PCR.

DISCUSSION

The results of this study revealed that fungal infection was proved in 17/30 (56.7%) of the enrolled immunocompromised patients with suspected fungal infection by panfungal PCR; 15/17 of these were missed by blood culture. Discrepancies in the results of culture and PCR are well known and have already been observed in previous studies.[13] 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.[14]

Badiee et al.[15] studied the microbiologic data of 310 immunocompromised patients (recipients who underwent transplantation and leukemic patients with neutropenia on chemotherapy) and compared them with molecular methods. Results of their study revealed that 10.6% of patients had positive cultures for fungal infections whereas 17.7% were positive for fungal infection by panfungal PCR. Another study compared results of culture, histology, galactomannan ELISA and real-time PCR in tissues and blood samples, and stated that 20 samples (64.5%) were positive by both culture and real-time PCR, 6 samples (19.4%) showed no growth of fungi but were positive by real-time PCR and, the remaining samples (16.1%) were negative by both culture and PCR. Worth mentioning is that all of the tissue samples were positive by both PCR and histology.[16]

According to the EORTC-MSG criteria, our patients at the outset of the study did not have possible (or proven) IFI. However, they were suspected of having fungal infection based on the existence of at least one host factor and the presence of fever or bronchopneumonia not responding to broad spectrum antibiotics. After performing mycological culture, 2 patients only turned out to have EORTC-MSG proven IFI when in fact another 15 did have PCR-proven IFI although they remained as not EORTC-MSG possible or probable, not to say proven IFI cases. This highlights the need for a more updated, patient and doctor-friendly criteria for diagnosis of IFI.

Landinger et al.[17] had focused on this 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 episodes in pediatric patients with high risk of IFI were investigated by panfungal PCR in his study, all proven, probable IFI, and all but one case with possible IFI were PCR positive. In 48 febrile episodes, patients received empirical antifungal on basis of clinical suspicion of IFI which did not meet any criteria of even possible fungal disease (EORTC IFI-ve). Twenty five of the episodes were positive by panfungal PCR; the sensitivity and NPV were in the range of 100%. In another study taking 2 consecutive positive results as the diagnostic criterion for IA, the sensitivity, specificity, PPV, and NPV of PCR were 91.6%, 94.4%, 73.3%, and 98.5%, respectively.[18] Flourent et al.[19] on the basis of the analysis of serum samples from 51 patients with IA as defined by the EORTC-MSG (4 proven, 29 probable, 18 possible), results of PCR-ELISA for Aspergillus was assessed in comparison with the results of high-resolution CT (HRCT), histological, and microbiological criteria as defined by the EORTC-MSG, and found that the sensitivity, specificity, PPV and NPV of

PCR-ELISA for proven and propable IFI were 63.6%, 89.7%, 63.6%, 89.7% respectively, while the combination of PCR-ELISA and galactomannan (GM) assay raised the sensitivity to 83.3%, increase the NPV to 97.6%, and decrease the specificity to 69.8%.

In the present study, patients were in an early stage of IFI. Hence, the time factor could offer another explanation for the observed differences in the results of blood culture and PCR. According to Estrella and coworkers [10] (18), fungal DNA was detected by PCR much earlier than GM in patients with aspergillosis and than HRCT detecting the lesions compatible with IFI. The mean time gain was 21 days compared with HRCT and 68 compared with GM.

The most common presentation in our PCR-proven IFI immunodeficient patients was fever not responding to broad spectrum antibiotics (82.4%). In the study of Kobayashi et al,[20] the most common infected region in 26 immunocompromised patients with IFI was the lung (54%), the liver (19%), while fungemia was present in 7.6%. Other researchers studied the most common presentation and found that in 44 patients with invasive candidiasis, 34 patients presented with candidemia, whereas among 10 patients with invasive aspergillosis, 8 patients presented with lung disease.[21]

Invasive aspergillosis predominantly affects patients with compromised phagocytic immunity, such as neutropenic cancer patients.[22] IA originating from colonized pneumatocele is also seen in patients with Jobs syndrome.[23] In our study, IA was seen in 2 SCID patients who had no neutropenia. However, all 2ry immunodeficiency patients with IA were neutropenic cancer patients. In the meantime, one third only of the neutropenic cancer children with PCR-proven fungal infection had IA; the rest had IC. Therefore, Candida is the most commonly isolated fungus in this group of patients.

Candida as a cause of fungal infection in immunocompromised patients in this study was more common than Aspergillus: 65% of patients were considered to have candidemia and 35% had IA. Badiee et al.[15] also found Candida in 72.2% and Aspergillus in 27.3% of their samples. Early diagnosis of disseminated candidiasis is a challenge since in one study only 35-50% of patients with antibiotic resistant neutropenic fever had positive blood cultures for Candida and radiologic tests had low specificity in that patient population.[24] In our study as well, 9 instances (30%) of PCR-proven candidemia were missed by blood culture which was also in agreement with the findings of Badiee et al.[15]

As a diagnostic tool in IA, culture is not reliable as it is a relatively slow, insensitive and requires a specialized expertise for species determination.[25] In the current study we found that patients who proved to have IA by PCR for Aspergillus had negative cultures for Aspergillus. This was in agreement with Gurtner et al.[16] who found that blood samples showed no growth of Aspergillus although Aspergillosis was confirmed by PCR and histology.

Out of the 17 patients diagnosed to have IFI by PCR, 14 were receiving antifungal treatment. In addition, the results of PCR did not differ significantly with the current intake of antifungal treatment. Mennink-Kersten et al.[26] analyzed the kinetics of the markers used in vitro for the diagnosis of IA. The authors showed that GM and other fungal antigens were released when Aspergillus was found in exponential growth phase, while fungal DNA was released when the hyphae break up, a phenomenon which occurs naturally by autolysis when the amount of nutrients is limited or when antifungal agents are present. In the study by Estrella and coworkers[18] the two patients in whom the infection was detected by PCR at the same time as radiology or GM testing were the only two patients who were not on itraconazole prophylaxis. This strengthens the hypothesis that increased autolysis after the introduction of the antifungal agent leads to increased DNA release.

Badiee et al.[15] reported that in patients treated with antifungal therapy, the fungal PCR assay became negative after 14 days if treatment was successful, and PCR-ELISA was positive until death if treatment fails. In our study PCR was positive in a patient treated with Amphotericin B for 14 days who was still feverish till the day of sampling. However, other studies reported low sensitivity of PCR for Aspergillus, in patients on antifungal treatment (amphotericin B for 14 days).[27,28]

Combined bacteremia and fungemia was detected in this study in 2 patients: the first patient diagnosed as SCID, had concurrent infection with *Staphylococcus aureus* and Aspergillus and the second patient with congenital neutropenia had both *Acinetobacter baumannii* and Candida infection. Concurrent bacteremia and fungemia had been previously reported in patients with candidemia.[29,30] The fungemia in these 2 patients was diagnosed only by PCR and was missed by blood culture. Perhaps the usual microbiological techniques may be inadequate to detect fungemia when concomitant bacteremia is present.[31]

In the current study, cases with IFI showed significantly increased CRP levels as compared to those without IFI. It has been reported that fever persisting at day 4 of admission, together with absolute monocytic count <100/mm^3 and a CRP greater than 90 significantly increased the risk for IFI in children with cancer.[32] Indeed there was no significant difference in the absolute neutrophil count between patients with and without fungal infection although it has been previously found that, in AML patients, the intensity of neutropenia can be a good predictor for IFI.[33]

In conclusion, real-time PCR proved superior to culture in the early diagnosis of IFI in patients with immunodeficiency before the appearance of the characteristic clinical and imaging signs. Reliance on blood culture alone at that stage would result in missing most of the positive cases with consequent delay in the initiation of specific treatment. Whether or not a negative PCR at that early stage of IFI could obviate the need for empiric antifungal treatment awaits further prospective studies.

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