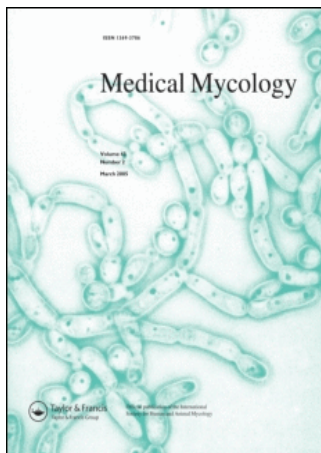


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Evaluation of pan-fungal PCR assay and *Aspergillus* antigen detection in the diagnosis of invasive fungal infections in high risk paediatric cancer patients

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Profound and prolonged neutropenia following chemotherapy is a major risk factor for systemic fungal infection. As the early diagnosis of invasive fungal infection (IFI) is difficult, these infections are still associated with high morbidity and mortality. Recently, Pan-fungal polymerase chain reaction (PCR) has been a promising aid in rapid, early diagnosis of IFI. During the past few years, increasing numbers of suspected IFIs were encountered at our institution in patients with prolonged neutropenia after intensified immunosuppressive chemotherapy. The aim of this study was to investigate the diagnostic utility of both the aspergillus galactomannan (GM) antigen and the pan-fungal PCR assay in the diagnosis of IFI in high risk febrile neutropenic paediatric cancer patients. During one year period, 91 febrile neutropenic (FN) paediatric cases at high risk for developing IFI while receiving chemotherapy were investigated at National Cancer Institute, Egypt. These patients were subjected to clinical evaluation, chest CT scan, conventional blood cultures for bacterial and fungal pathogens, aspergillus GM antigen detection and PCR assay utilizing pan-fungal primers. Of the 91 FN episodes, 15 were proven IFI; whereas 27 cases were either probable ($n=13$) or possible IFI ($n=14$), and 49 were unlikely to be IFI episodes. Based on positive results for proven/probable IFI and compared to culture results, Pan-fungal PCR showed sensitivity, specificity, positive and negative predictive values of 75%, 92%, 84% and 87%; respectively. Aspergillus antigen test showed a sensitivity of 79%, specificity of 61%, positive and negative predictive values of 54% and 83%; respectively. A negative PCR in the proven and probable cases was closely related to previous antifungal therapy for a prior history of IFI. In patients at high risk for IFI, neither the sensitivity, nor specificity of the GM test was sufficient. The results of PCR assay was reasonably specific but not very sensitive and had a chance of missing the diagnosis of IFI. The PCR assay seems a promising test for objectively defining IFI, but is not recommended as the only tool for diagnosing IFI. Combining microscopy, culture, and PCR may improve the diagnostic outcome.

Keywords invasive fungal infection, haemato-oncology patients, polymerase chain reaction, galactomannan, aspergillus antigen

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Introduction

Invasive fungal infection is a serious opportunistic infection in the immunocompromised host, especially allogeneic bone marrow transplant recipients, solid

organ transplant recipients and patients receiving cytoreductive or marrow ablative therapy for haematological malignancies with the resultant severe neutropenia. Because of the difficulty in diagnosing the infection early in the course of the disease, IFI is associated with considerable morbidity and mortality [1]. Cultures are often negative and positive results may require days or weeks to grow and need a careful evaluation to determine clinical importance. Moreover, the histological examination of tissue specimens obtained by invasive procedures, which is still considered the gold standard, is often precluded by profound cytopenia. Both *Aspergillus* and *Candida* species are the most common fungal pathogens in this population of patients, but as invasive aspergillosis (IA) is the most serious opportunistic infection in the immunocompromised host nowadays, more attention is directed to IA [2].

Early diagnosis of invasive aspergillosis is critical for a good outcome. Unfortunately, diagnosis is difficult and is often delayed. Clinical signs indicating a definite diagnosis of fungal infection is frequently absent in cancer patients with fever not responding to appropriate broad spectrum antibacterial agents the most common and frequently the only manifestation of the disease. Axial computerized tomography is helpful diagnostically in neutropenic patients when the halo sign is present but this finding is non specific and is not present in all patients. Definitive diagnosis often requires an invasive procedure, such as open lung biopsy, which may be problematic because of the critical condition of the patient, while a delay in initiating antifungal therapy, or conversely, a hasty use of empiric or prophylactic amphotericin B before making a definitive diagnosis may result in treatment failure for full-blown infection or excess toxicity; respectively [3].

In recent years, progress has been made in the development and evaluation of sensitive serodiagnostic assays, including detection of genomic DNA sequences and fungal antigens, which appears to be promising in rapid and early diagnosis of IFI [4]. However, the sensitivity and specificity of the assays vary considerably in different studies, depending on several factors including patient selection, and clinical application of the test, and issues regarding the release and circulation of galactomannan and fungal DNA remain to be clarified [5]. With the increasing use of aggressive chemotherapy causing profound and prolonged depressed immunity, the risk of invasive mycoses have increased. At our institution, fungal infections have been reported to constitute 3.7% of febrile neutropenic episodes [6], but lately, increasing numbers of suspected IFIs are encountered in patients with prolonged

neutropenia after intensified immunosuppressive chemotherapy. Therefore, early detection is crucial for proper management of patients at risk of IFI. Thus, we attempted to evaluate the PCR assay and the aspergillus antigen detection as compared to conventional microbiological procedures in the early diagnosis of IFI.

Patients and methods

Patient selection

Patients were selected from paediatric patients admitted to the department of paediatrics for their cancer chemotherapy at National Cancer Institute, Cairo University. Febrile neutropenic paediatric cancer patients who are at risk of developing systemic fungal infections were prospectively studied during a one year period extending from April 2003 to April 2004. Patients were included in the study if essentially they had fever not responding to empirical antibiotics at 96 h. Together with fever, they had any of the following risk factors: prolonged neutropenia (less than $0.5 \times 10^9/l$ for 7 days or more), steroid therapy for more than 10 days or lower respiratory infection developing within one month after completion of chemotherapy. During the period of the study, 91 paediatric patients fulfilled the criteria of being high risk of developing systemic fungal infection. Patients were subjected for diagnostic work-up including complete clinical assessment for any sign of fungal infection and high resolution pulmonary computerized tomography (CT) scan was performed in patients with febrile episodes extending for more than one week.

Culture

Two blood culture sets were drawn from each patient within the first day of inclusion in the study, from two separate veins. Collected blood was injected directly into Bactec® (Becton Dickinson, USA) culture vials. Vials were incubated in the Bactec® 9050 incubator after collection. Identification of isolates was carried out utilizing Sensititre AP80 and AP90 auto-identification plates (AccuMed International Ltd. Interhorne Lane, East Grinstead, West Sussex, UK) for Gram-negative and Gram-positive organisms, respectively. If a localized site of infection was clinically suspected to be the source of infection, a sample was obtained accordingly, in addition to the peripheral vein samples for blood cultures. Repeated culturing was performed for patients not responding to appropriate broad spectrum antibiotic therapy.

Positive blood cultures and other samples were subjected to routine culture for fungi, by being plated onto Sabouraud dextrose agar at room temperature. Direct examination of specimens was done whenever possible. Fungal growth was identified by the culture characteristics and the morphologies of the conidiphore and conidia. Microscopic examination of specimens was performed using lactophenol cotton blue.

Detection of soluble Aspergillus antigens

Serum from selected patients were obtained and the test was performed using the Pastorex Aspergillus kit (BIO-RAD, Marnes-la-Coquette, France) [7].

PCR assay

Serum samples were treated with a sorbitol buffer containing 200 units of lyticase (Sigma L4205) to disrupt fungal cell wall. After incubation at 35°C for 1 h, fungal spheroplasts were precipitated by centrifugation at 15000 × *g* for 10 min. The supernatant was discarded and the pellet resuspended in 400 µl of PBS buffer and 20 µl of proteinase K (Qiagen) and 200 µl of buffer AL (QIAmp DNA Mini Kit and QIAmp DNA Blood Mini Kit, Germany) then incubated at 56°C for 10 min. Genes segments of the 18S rRNA of various fungal pathogens were amplified by PCR using universal primers [8]. Primers (5'-ATT GGA GGG CAA GTC TGG TG and 5'-CCT ATC CCT AGT CGG CAT AG) bind to conserved regions of the fungal 18S small-subunit rRNA.

The PCR protocol was performed in the following sequence, one cycle of denaturing at 94°C for 3 min., followed by 30 cycles of denaturing at 94°C for 30 seconds, annealing at 58°C for 1 min and extension at 72°C for 1 min, then one final extension at 72°C for 7 min. All PCR runs included a positive control (containing DNA of one of the fungal isolates) and a negative control (water in place of DNA). Detection of product: An aliquot of 15 µl of the PCR product was run on a 2% agarose gel and stained with ethidium bromide. The predicted product size was 450 base pair. The PCR assay was run by an investigator blinded to clinical and microbiological data.

Case definitions and stratification of patients into groups

The patients were stratified into groups according to the criteria defined by the European Organization for Research and Treatment of Cancer (EORTC) [9] for IFI. Proven IFIs implied the histological evidence of tissue invasion by filamentous fungi in specimens

obtained by biopsy or autopsy or a positive culture for fungi from a normally sterile body fluid obtained in a sterile procedure, as well as clinical or radiological symptoms consistent with infection. Probable IFIs was defined by the presence of at least 1 host factor criterion i.e fever, neutropenia, long steroid therapy; and 1 microbiological criterion, i.e., positive culture or cytologic/direct microscopic evaluation of fungi and 1 major, i.e., radiological evidence (or 2 minor) clinical criteria from abnormal site consistent with infection. Possible IFIs was defined as fever not responding to 5 days of adequate broad spectrum antimicrobials or relapsing after initial defervescence in persistently neutropenic patients or in patients receiving high dose corticosteroids and 1 microbiological or 1 major, or 2 minor, clinical criteria from abnormal site consistent with infection. Unlikely IFI, referred to patients with neither microbiological nor clinical evidence of IFI.

Antifungal therapy

Amphotericin B was added for all patients with fever refractory to 7 days of broad spectrum antibacterial coverage and to those developing recurrent fever while still neutropenic; and was added earlier than day 7 to cases with a previous history of IFI.

Statistical analysis

Data management and analysis were performed using Statistical Analysis Systems. Numerical data were summarized as means and standard deviations, categorical measurements as percentages. To measure the differences between groups, a two-way analysis of variance (ANOVA) with repeated measures on one factor was performed. Agreement between tests was measured by the Kappa. All *p*-values are two-sided and *p*-values ≤ 0.05 were considered significant. The objective of this study was to assess the diagnostic potential of both the GM test and PCR in a consecutive cohort of paediatric haematology-oncology patients at high risk for IFI. As previously described [10], we assumed that all episodes of proven and probable IFI were true-positive episodes and that the non-IFI episodes were true-negative ones.

Results

Out of all cancer patients experiencing febrile episodes from April 2003 to April 2004, 91 cases fulfilled our inclusion criteria. Their age ranged from 2 to 18 years with a mean of 8 ± 4.8 years and a median of 6 years. Thirty-seven cases (41%) were diagnosed as acute lymphoblastic leukaemia (ALL), 34 (37%) acute

myeloid leukaemia AML, 9 (10%) non-Hodgkin's lymphoma (NHL) and 11 (12%) were diagnosed as solid tumours. Clinically, fever not responding to empirical antibiotics was the only presenting sign in 43 cases, whereas lower respiratory tract infection (LRTI), diarrhoea, skin infection, cannula site infection, oesophagitis and sinusitis were encountered in 34, 5, 4, 2, 2 and 1 of the cases; respectively.

Blood specimens for blood cultures were obtained routinely for all patients. Other samples were bronchoalveolar lavage fluid, nasal discharge, sputum and wound swabs. Fungi were recovered from cultures in 28 specimens, which were *Aspergillus* species in 11, *Candida* species in 7, and *Penicillium marneffei* in 8 specimens, *Mucor* and *Histoplasma capsulatum* each in one case. Pure fungal growth was encountered in 12 cases, while mixed bacterial and fungal growth was encountered in the remaining cases. The majority of cultures were positive on repeated culturing in cases not responding to appropriate broad spectrum antibiotics. Eighty-eight patients underwent CT scans of the chest. Thirty-one patients showed positive findings suggestive of fungal infection by CT chest, whereas 57 patients had normal CT findings. A previous fungal infection, with positive CT findings suggestive of IFI, was reported in 6 patients enrolled in the present study.

Fifty-four out of 91 patients (59%) were found to be positive when tested for the *Aspergillus* antigen in serum using the Galactomannan test; whereas, 32 patients (35%) were found to be PCR positive. Figure 1 illustrates the resultant PCR product. The results of both tests among the different groups of patients are summarized in Table 1. The PCR positivity preceded culture results in the majority of cases, as PCR assay was performed usually at day 4 of the episode; whereas, repeated culturing was usually performed to get a positive result in patients with persistent FN in spite of being on broad spectrum antibiotics. The sensitivity, specificity, positive and negative predictive values of GM antigen and PCR tests are summarized in Table 2 together with the 95% confidence intervals.

Fifteen (16.5%) patients were considered proven IFIs, 13 (14.3%) patients were reported as probable IFIs, 14 (15.4%) were possible to have IFIs and 49 (53.8%) were considered unlikely to be IFI, or non-IFI episodes. Of the PCR negative patients within the proven and probable cases, 6 were reported to have previous IFI with positive CT findings suggestive of IFI and had received Amphotericin B for more than 2 weeks beforehand.

Four out of the 32 PCR positive patients (8.3%), neither developed clinical signs of IFI, nor received empirical antifungal treatment, i.e., false positive

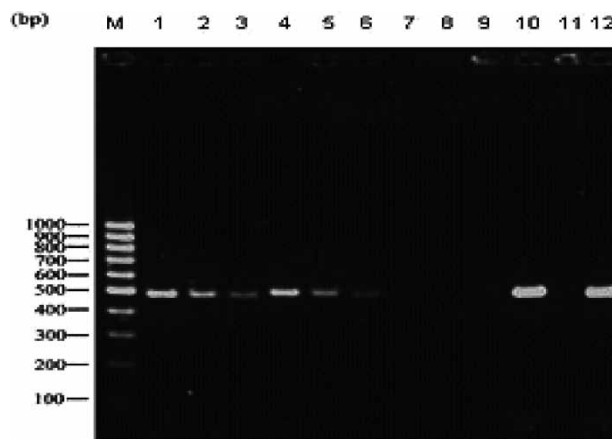


Fig. 1 Gel electrophoresis showing fungal DNA at 450 bp; lane M is for molecular weight marker, while lanes 1–10 demonstrate PCR products of patients; lanes 1–6, and 10 are positive cases, and lanes 7–9 are negative cases. Lane 11 is for the negative control, while lane 12 is for the positive control which was DNA extracted from *Candida*.

results. Thus, only 4 of the 49 patients without clinical evidence of an IFI were found to be PCR positive.

Relation of the PCR results with clinical findings

Clinically, 12 of the proven IFI cases had documented lower respiratory tract infection (LRTI) with a CT picture suggestive of fungal infection, one case of documented sinusitis, a case of oesophagitis and one patient with unexplained fever who subsequently developed culture proven candidemia. Thirteen patients were proven by positive cultures; whereas, two patients were histologically proven to be IFI after death. An unfavourable outcome was encountered in 4 cases of the proven IFI who were all PCR positive, early in their course of infection. Of the probable IFI patients, 10 manifested with documented LRTI, one case manifesting with extensive bed sores, one case of oesophagitis, and a case of unexplainable fever. Four of the five cases with an unfavourable outcome in this group were PCR positive. As regards the possible IFI cases, eight manifested with documented LRTI, one case with perianal skin infection, one case of refractory gastroenteritis and 4 cases with unexplainable fever. The remaining patients ($n=49$), cases who were unlikely to be IFI, mainly manifested by unexplainable fever ($n=37$). Three of the latter group showed signs of mild LRTI, but their CT were not suggestive of fungal infection.

Table 1 The results of GM *Aspergillus* antigen test and PCR assay among the different stratified groups in relation to IFI in the 91 studied paediatric cancer patients

	<i>n</i> (%)	PCR <i>n</i> (%)	Antigen <i>n</i> (%)	Dead <i>n</i> (%)
Proven	15 (16.5)	13 (86.6)	13 (86.6)	4 (26.6)
Probable	13 (14.3)	8 (61.5)	9 (69.2)	5 (38.5)
Possible	14 (15.4)	7 (50.0)	13 (92.8)	3 (21.4)
Unlikely IFI	49 (53.8)	4 (8.3)	19 (38.8)	5 (10.2)

Discussion

Conventional laboratory diagnostic methods for invasive fungal infection in haemato-oncology patients are insensitive, resulting in late diagnosis and contributing to high mortality. In recent years, progress has been made in the development and evaluation of sensitive serodiagnostic assays, including detection of genomic DNA sequences and fungal antigens, which aid in a rapid, early diagnosis of IFI. The sensitivity and specificity of the assays vary considerably between studies, highlighting the need to correlate serological results with conventional laboratory tests and clinical or radiological findings and favoring their use to screen high risk patients [3]. Thus, in this study we compared the diagnostic potential of different laboratory methods used to screen IFI in a prospective setting where the panfungal PCR assay and detection of Galactomannan antigen were done in a cohort of high-risk paediatric cancer patients. The patients were classified according to IFI diagnosis criteria of European Organization for Research and Treatment of Cancer/Mycosis Study Group (EORTC/MSG) [9]. Of the 91 patients fulfilling the criteria of inclusion in the present study, being at high risk for IFI, 15 were proven IFI, 13 probable, 14 possible IFI and 49 were unlikely to have IFI episodes.

The use of panfungal PCR assays has opened a new era in mycology diagnostics [8]. It can be used to detect a broad range of fungal pathogens and can be combined with species identification. Another advantage of this assay is that DNA can be detected with high sensitivity and specificity when performed from sterile sites such as blood [11]. Thus, serum based PCR may be especially valuable in our paediatric group of patients in whom invasive procedures are hazardous. Moreover, the panfungal PCR assay allows detection of

fungal infections earlier, with a time gain of 1–8 days than conventional cultures [8].

In the present study, panfungal PCR showed a sensitivity of 75%, a specificity of 92%, positive and negative predictive values of 84% and of 87%; respectively. Yet, 87% of the proven IFI cases were positive by PCR. In addition, all patients with proven IFI with an unfavourable outcome were PCR positive. A reported sensitivity, specificity, positive predictive and negative predictive values for PCR of 55%, 93%, 40% and 96%; respectively was reported in the diagnosis of IA [12]. Recently, the sequential positive results of panfungal PCR in proven/probable IFI cases, sensitivity, specificity, positive and negative predictive values of 75%, 70%, 15% and 98%, respectively, were recorded [13]. The limited sensitivity of the PCR assay in some studies could be related to the fact that the statistical parameters of a diagnostic test can be dramatically affected when there is some uncertainty regarding the disease status, especially in the probable/possible cases. Although several authors have also reported excellent sensitivity in PCR assays for IA [14,15], yet we cannot directly compare those results with ours since there were differences in the target genes, methods of DNA extraction, and designs of the PCR amplifications. Some form of standardization is required to make an international comparison possible.

PCR results were related to the clinical findings in the present study, as 12 of the proven and 10 of the probable IFI cases had a documented LRTI with suggestive CT findings. Similar prospective studies confirmed the utility of screening patients at high risk of IFI using PCR assays [15,16]. These findings point to the potential clinical utility of this method in directing therapeutic strategies for IFI. In the study by Hebart *et al.*, all patients developing proven IFI

Table 2 The sensitivity, specificity, negative and positive predictive values of GM *Aspergillus* antigen test and PCR assay in the diagnosis of IFI in 91 paediatric cancer patients

Test	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	Positive predictive (%) (95% CI)	Negative predictive (%) (95% CI)
PCR	75 (55–87)	92 (80–97)	84 (63–95)	87 (74–94)
Antigen	79 (59–90)	61 (46–74)	54 (38–69)	83 (67–93)

were PCR positive, and PCR was found to be the earliest indicator of invasive fungal infection preceding clinical evidence by a mean of 5.75 d (range 0–14 days). In febrile neutropenic patients without a prior history of invasive fungal infection, a sensitivity of 100% and a specificity of 73% of the PCR assay for the development of proven or probable IFI were documented [15].

Sensitivity and specificity values are encouraging and PCR positivity precedes the development of clinical and radiological signs and administration of empirical antifungal therapy in most patients. Unfortunately, the occurrence of false positive results is expected and is reflected in the modest positive predictive values recorded in published studies [3]. In agreement, 4 patients in the current study who showed no evidence of fungal infection were PCR positive (Non-IFI episodes). However, these false positives may reflect sub-clinical infection masked by empirical therapy or neutrophil recovery [3]. Commenting on PCR positivity which was never associated with IFI and resolved without antifungal treatment, it is possible that these short PCR-positive episodes result from subclinical aspergillosis, fungal colonization or from macrophages releasing DNA measurable by PCR [16].

However, 7 of the proven and probable groups, in the present study, were negative by PCR. Of these patients, 6 were reported to have previous IFI with positive CT findings suggestive of IFI and had received Amphotericin B for more than 2 weeks beforehand. Similar results were previously reported [4,16], and it was stated that inhibition of the PCR in the presence of residual amphotericin B could be responsible for such results. Another possible explanation could be the amount of fungal DNA available in serum was very low. It was previously concluded that the ability to detect free fungal DNA is dose dependent; in addition, the sensitivity of the plasma PCR is lower than that of the PCR performed on whole blood samples [17].

In the present study, latex agglutination (LA) test was used for the detection of GM aspergillus antigen and was positive in 13, 9, 13 and 19 of the proven, probable, possible cases and unlikely IFI episodes; respectively with a sensitivity of 79%, specificity of 61%, and positive and negative predictive values of 54% and 83%; respectively. Previous evaluation of the GM test in patients with neutropenia showed variable sensitivities ranging from 30% to 95% [7]. The primary limitation of GM in our study was the false positive reactions, which was reported to be higher in children; specificity was 48% versus 98% in adults [1]. It is now well established that this test gives false positive results, especially in children, due to possible passage through the intestinal mucosa of GM present in milk, rice, or

rich protein nutrients [18]. Even with EIA, false positive results occur and remain obscured, whether this positivity results from sub-clinical aspergillosis or intestinal fungal colonization [19].

In conclusion, the pan-fungal PCR assay in serum samples correlated well with the clinical findings of invasive fungal infection. However, some findings are ambiguous and confirm the difficulties in diagnosing invasive fungal infection. So far, a clear benefit of the use of PCR is the identification of cases in which culture results are negative. The PCR test is more specific than the GM test for screening high risk group cancer patients in a hospital laboratory. It may be most useful as a screening method in high-risk patients, either to direct early pre-emptive antifungal therapy or to determine when empirical antifungal therapy can be withheld in patients with antibiotic-resistant neutropenic fever. However, these strategies require further assessment for their implications on selection of patients at risk of IFIs who will benefit from early initiation of antifungal therapy. Combining microscopy, culture, and PCR may improve the diagnostic outcome.

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