Utility of Panfungal PCR in the diagnosis of invasive fungal infections in febrile neutropenia

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Abstract

Background: The prevalence of invasive fungal infections (IFIs) is increasing due to the increasing population of immunocompromised patients. Fungal culture is the gold standard for diagnosis but not sensitive and the turnaround time is long. Samples for histopathology are difficult to obtain because of profound cytopenias. We conducted this study with the aim to evaluate panfungal PCR for the diagnosis of IFIs in patients of febrile neutropenia. **Methods:** This was a single-centre, cross-sectional observational study. Patients of febrile neutropenia suspected of having IFI were included in the study. Panfungal PCR was performed on the blood of included patients along with other investigations for diagnosis of IFI. The sensitivity, specificity, positive predictive value, and negative predictive value of panfungal PCR were calculated using EORTC/MSG 2008 criteria as the gold standard. **Results:** Fifty patients of febrile neutropenia were included in the study, of which 52% were diagnosed positive by panfungal PCR assay. The sensitivity, specificity, positive predictive value, and negative predictive value of panfungal PCR assay was found to be 82.76%, 90.48%, 92.31% and 79.17% respectively. **Conclusion:** Panfungal PCR is a promising and highly sensitive diagnostic test for screening at-risk patients suspected to have IFIs. The use of panfungal PCR assay in combination with other diagnostic modalities and clinical judgment can be very helpful in the early diagnosis of IFI.

Keywords: Invasive fungal infections, neutropenia, panfungal PCR

Introduction

Invasive fungal infections (IFIs) are infections where fungi invade deep into the tissues and result in prolonged illnesses.[1] Histopathological identification and fungal culture are the usual means of diagnosis of IFIs. However, patients at risk for IFIs are also at high risk for complications associated with invasive biopsies due to profound cytopenias.[2] Blood cultures are often negative in >50% of patients with documented disease and cause a considerable diagnostic delay.[3]

Nucleic acid–based diagnostic techniques are the fastest growing and most-promising segment of fungal diagnostics. These methods can detect IFIs with high sensitivity (approaching 100% in some studies). In many cases, fungal PCR was positive several days before fungal pathogens like *Candida* could be detected by blood culture.[4]

There are few reports of panfungal PCR assays targeting multiple fungal genera.[5,6] However, the sensitivity and specificity of the assays vary considerably in the studies. The studies depicting the analytical ability of real-time panfungal PCR to detect IFIs as per the European Organization for Research and Treatment of Cancer (EORTC/MSG) guidelines are fewer.[4]

To the best of our knowledge, none of the panfungal PCR assays are currently approved by FDA. Species-specific *Aspergillus* and *Candida* PCR have only recently been included in the revised EORTC/MSG 2019 diagnostic criteria for IFI.[7]

The aim of our study was to evaluate the utility of panfungal PCR assay in a group of patients of febrile neutropenia with

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suspected IFI and to correlate the results with the EORTC-MSG diagnostic criteria for invasive fungal infections. As our study was conducted before 2019, EORTC/MSG 2008 criteria were used.

Methods

The study was a single-centre, cross-sectional observational study conducted in a North Indian hospital. Approval from the Institutional Ethical Committee and a written informed consent from all the subjects enrolled into the study were obtained. Patients aged more than 18 years and having fever of any duration and neutropenia were included in the study. Fever was defined as a single oral temperature >38.5^0 C or three temperatures of >38^0 C at least 4 hours apart in a 24-hour period. Neutropenia was defined as <500 neutrophils/cu.mm or <1000 neutrophils/cu.mm and predicted to decline to <500/cu.mm over the next 48 hours. Patients already receiving betalactam antibiotics (give false-positive fungal PCR) or systemic antifungal therapy were excluded from the study.

The subjects underwent the following investigations: complete haemogram, urine for routine and microscopic examination, chest X-ray, kidney function tests, liver function tests, random blood sugar, blood, urine and sputum for fungal culture and sensitivity, blood for galactomannan antigen test (GM ELISA) and blood for panfungal PCR assay. Broncho-alveolar lavage (BAL) for fungal culture and sensitivity and HRCT chest were done where clinically indicated. Patients were treated as per the standard guidelines for febrile neutropenia. They received broad-spectrum antibiotics like third-generation cephalosporins on the first day. Those who remained febrile despite three days of appropriate antibiotics were then started on therapeutic antifungal drugs. Additional samples were sent for microbiological investigations to establish a definitive diagnosis if needed. On the basis of history, physical examination and investigations, the patients were then categorised as per EORTC-MSG 2008 criteria [Annexure 1] into the following three categories:
Proven IFI
Probable IFI
Possible IFI
Panfungal PCR methodology.

Whole blood (5 ml) samples were collected in an EDTA vacutainer for PCR assay. Whole blood (5 ml) in a plain vacutainer was collected for serum galactomannan antigen detection. All blood samples were stored at − 80^0 C until DNA extraction.

Panfungal PCR kit: The panfungal PCR assay evaluated in our study was done using GenoSen's Real Time PCR kit. It can detect a total of 16 fungal species (six species of *Aspergillus*, nine species of *Candida* and one species of *Fusarium*) [Table 1]. For sensitivity, a dilution series was setup from 10^6 down to 10^0 copies/μl of panfungal DNA and analysed with a Panfungal Real Time PCR Kit. The detection limit as determined for Panfungal Real Time PCR Kit was 50 copies/ml.

Table 1: Fungal species detected by the panfungal real-time PCR assay
1. *Aspergillus fumigates*
2. *Aspergillus flavus*
3. *Aspergillus terreus*
4. *Aspergillus versicolor*
5. *Aspergillus nidularis*
6. *Aspergillus clavatus*
7. *Fusariun solani*
8. *Candida tropicalis*
9. *Candida glabrata*
10. *Candida crusei*
11. *Candida albicans*
12. *Candida parapsilosis*
13. *Candida guilliermondii*
14. *Candida kefyr*
15. *Candida lipolytica*
16. *Candida lusitaniae*

DNA extraction kit: Qiagen QIAamp DNA Mini extraction kit.

The real-time PCR instrument: Rotor Gene™ 6000, Corbett Research (Australia)

Reference fungal strains: The ability of the panfungal PCR assay to detect all fungi species of interest was determined by testing DNA derived from cultures of reference strains. The panfungal PCR assay performed in our study exploits the Taqman principle targeting the 18S ribosomal RNA gene.

DNA extraction from the blood samples and ATCC fungal strains were performed using the Qiagen QIAamp DNA Mini extraction kit. For effective lyses of fungal cell walls, the heat shock method (the samples were incubated at 100^0 C in a water bath for 3 min and then transferred to liquid nitrogen for 1 min) was incorporated in addition to the Qiagen protocol. The DNA extraction protocol was standardised using reference strains and blood spiked with the desired fungal load.

Statistical analysis

Observations were recorded in standard proforma. Categorical variables were presented in number and percentage (%) and continuous variables were presented as mean ± standard and median. The results of panfungal PCR analysis in relation to the proven, probable and possible IFI criteria by the EORTC/MSG 2008 were used for the calculation of the sensitivity, specificity, negative predictive value (NPV), and positive predictive value (PPV) of the assay. The degree of agreement was quantified using kappa statistics. The association of clinical and radiological features with PCR results was assessed by Chi square test/Fischer test whichever was appropriate. A *P* value of < 0.05 was considered significant. Conclusions were drawn based on the results obtained.

Results

Fifty patients with febrile neutropenia were enrolled in the study. The age of the patients ranged from 19 years to 40 years,

with a mean age of 27 years. The study population had 86% males (43/50) and 14% females (7/50). Of the 50 patients enrolled into the study, 36% patients (18/50) had acute myelocytic leukaemia (AML), 28% cases (14/50) had acute lymphocytic leukaemia (ALL), 30% cases (15/50) had aplastic anaemia and 6% cases (3/50) had underlying lymphoma. The patients were divided into four groups according to the EORTC/MSG 2008 criteria. Groups A, B, C and D consist of patients with no IFI, proven IFI, probable IFI and possible IFI, respectively. After evaluation and investigations, three patients were in the proven IFI group, 22 patients were in the probable IFI group while possible IFI group had four patients and 21 patients were in the no IFI group.

The results of the panfungal PCR assay evaluated in this study among the patient groups are shown in Table 2. Fifty two percent patients (26/50) were diagnosed positive by panfungal PCR assay while the remaining 48% patients (24/50) were diagnosed negative by panfungal PCR assay. When the results of panfungal PCR assay were evaluated in the individual patient groups, the following observations were made: In Group A, i.e., patients without any evidence of IFI according to the EORTC/MSG criteria; 9.5% patients (2/21) tested positive by the panfungal PCR assay, while 90.5% patients (19/21) tested negative. In Group B (Proven IFI), 66.7% patients (2/3) tested positive by the panfungal PCR assay, while 33.3% patients (1/3) tested negative. In Group C (Probable IFI), 81.8% patients (18/22) tested positive by the panfungal PCR assay, while 18.2% patients (4/22) tested negative. In Group D (possible IFI), 100% patients (4/4) tested positive by the panfungal PCR assay, while none tested negative.

Using EORTC/MSG 2008 criteria as the 'gold standard' for defining IFI, the sensitivity, specificity, PPV, NPV and likelihood ratios of panfungal PCR assay was calculated. The sensitivity, specificity, PPV and NPV of panfungal PCR assay was 82.76%, 90.48%, 92.31% and 79.17%, respectively. The positive likelihood ratio and negative likelihood ratio of panfungal PCR assay in the study was 8.69 and 0.19, respectively. The diagnostic accuracy of the panfungal PCR assay evaluated in the study was 86% [Table 3]. The degree of agreement was quantified using 'Kappa statistics. The kappa value observed was 0.718 with a standard error of 0.098 and 95% confidence interval from 0.526 to 0.910. The strength of agreement is considered to be 'good'.

The strength of association between the results of panfungal PCR assay and the presence or absence of clinical/mycological criteria in the study subjects was evaluated [Table 4]. The association between the clinical criteria and panfungal PCR results as assessed by Fischer's exact test was statistically significant with a two-tailed *P* value of < 0.0001. The association between mycological criteria and panfungal PCR results as assessed by Fischer's exact test was statistically significant with a two-tailed *P* value of 0.0002.

Discussion

Our study was undertaken to evaluate the role of panfungal PCR assay in the diagnosis of IFIs. Panfungal PCR was performed

Table 2: Panfungal PCR results in different patient groups according to the EORTC/MSG 2008 criteria

PCR results all episodes (*n*=50)	No IFI *n* (%)	Proven IFI *n* (%)	Probable IFI *n* (%)	Possible IFI *n* (%)
PCR positive (*n*=26)	2 (9.5%)	2 (66.7%)	18 (81.8%)	4 (100%)
PCR negative (*n*=24)	19 (90.5%)	1 (33.3%)	4 (18.2%)	0 (0%)
Total	21	3	22	4

PCR: polymerase chain reaction ; IFI: invasive fungal infection

Table 3: Sensitivity, specificity, positive predictive value, negative predictive value, likelihood ratios and diagnostic accuracy of panfungal PCR assay in diagnosing invasive fungal infections using the EORTC/MSG 2008 criteria as gold standard

Parameter	Value (95% confidence interval)
Sensitivity (95% confidence interval)	82.76% (64.21%-94.09%)
Specificity (95% confidence interval)	90.48% (69.58%-98.55%)
Positive predictive value (95% confidence interval)	92.31% (74.83%-98.83%)
Negative predictive value (95% confidence interval)	77.78% (57.84%-92.79%)
Positive likelihood ratio (95% confidence interval)	8.69 (2.30-32.81)
Negative likelihood ratio (95% confidence interval)	0.19 (0.08-0.43)
Diagnostic accuracy	86%

Table 4: Association between the panfungal PCR results and the clinical and mycological criteria based on the EORTC/MSG 2008 criteria for invasive fungal infections

	PCR positive (*n*%)	PCR negative (*n*%)	*P*
Clinical criteria			
Present (*n*=27)	23 (85.2%)	4 (14.8%)	<0.0001
Absent (*n*=23)	3 (13%)	20 (87%)	
Mycological Criteria			
Present (*n*=25)	20 (80%)	5 (20%)	
Absent (*n*=25)	6 (24%)	19 (76%)	0.0002

on the blood samples of immunocompromised and neutropenic patients suspected of having IFI and the diagnostic performance of the assay was studied in comparison to the EORTC/MSG 2008 criteria. In our study, there were only three cases of proven IFI as per the EORTC/MSG criteria with the *Candida* species being cultured from blood in them. This once again shows that blood cultures are often negative owing to the multiplication of yeasts and moulds in internal organs like liver and spleen.[8] All the three cases of candidemia in our study were caused by non-albicans candida species. This is consistent with the reports of the changing epidemiology of invasive candidiasis as reported by Pfaller *et al.*[9] and Chakrabarti A *et al.*[10] from India. *Aspergillus* species were not isolated from the blood of any patient.

The sensitivity, specificity, PPV, and NPV of panfungal PCR was established in our study by comparing the group of patients who had no evidence of IFI with those who had IFI as per the EORTC/MSG 2008 criteria. The sensitivity and specificity in

our study was 82.76% and 90.48%, respectively, while the PPV and NPV in our study was 92.31% and 77.78% respectively. These values are consistent with those reported in most of the earlier studies.

GuoJun Cao1 *et al.*[11] in their study demonstrated panfungal PCR to have a sensitivity and specificity of 82.8% and 79.8%, respectively, for probable IFDs, and a sensitivity and specificity of 87.5% and 71.4%, respectively, for a combination of probable and possible IFDs. Capoor *et al.*[4] found that for proven IFI, the sensitivity and specificity of the panfungal PCR assay were 94.3% and 95.2%, respectively with a PPV of 97.6% and NPV of 88.9%. Various authors have reported the sensitivity of panfungal PCR in the diagnosis of IFI in the range of 50–100% (Deshpande *et al.* 2011,[12] Gomez *et al.* 2017,[13] Lass Florl *et. al.* 2013,[14] Gupta P et. al. 2016[15], El-Ashry MA *et al*[16]). Ala Houhala *et al.* found a concordance of 85% between PCR and the conventional methods of culture and microscopy.[17] A wide range of sensitivity seen in these studies may be due to different case definitions and criteria for a PCR positive episode.

Very few studies have been reported in India on the role of fungal PCR in invasive fungal infections. We could find only four such studies.[4,10,12,15]

In our study, false positive results were observed in two out of 50 patients evaluated. As observed by several other authors, these false positive results could be due to subclinical infection, fungal colonisation, contamination by airborne fungal spores, fungal PCR product carryover and cross reactivity with non-fungal DNA. As Taqman technology was used in our study, there is little risk of false positivity due to the amplified products in the previous cycles. The risk of contamination is also low as the positive material (specimens, standards or amplicons) were stored separately from all other reagents. All workstations were wiped with 5% hypochlorite and 70% ethanol at every stage. The extraction of DNA, preparation of master mix, addition of template and amplification were carried out in biosafety hoods in separate laboratories. All the reagents including the NTC (except for standards and specimens) were mixed and dispensed in a pre-mix area.

Jordanides *et al.* had commented on the difficulty in determining an actual 'false-positive' result from an early 'true positive' result, reflecting the fact that PCR may be a more sensitive indicator of early IFI.[18]

In our study, false negative results were observed in five out of the 50 patients evaluated. This may be because many cases of probable and possible IFIs may be due to non-fungal causes as has been observed previously by Jordanides *et al.*[18] Maertens *et al.* demonstrated, after incorporation of autopsy data, that 12/43 patients who had possible IFI based on the EORTC/MSG criteria actually had definite IFI, whereas the remaining 31 cases had no evidence of IFI.[19] This supports the suggestion that an aetiology other than IFI may be responsible for the clinical signs

and symptoms in those five patients who came out to be falsely negative by PCR. Another possible reason for the PCR negativity may the higher levels of fungal DNA detection limit in our study. We had set a limit of 50 CFU/ml while most of the other studies had detection limits as low as 10–20 CFU/ml. Also, it is worth mentioning that the PCR assay done in our study could detect only a total of 16 fungal species and there may have been cases of IFI which were caused by species that was not detected by the PCR. In addition, in our study, samples for panfungal PCR were taken only once at the onset of fever and no subsequent samples were taken. This might have led to the false negative reports in some of our cases as sequential positive PCR reports have been shown to increase the sensitivity of PCR in the studies by Landlinger *et al.*[5]

Our study demonstrated the association between the results of panfungal PCR and the clinical and mycological criteria of the patients. The association between clinical criteria and panfungal PCR results as assessed by Fischer's exact test is statistically significant with a two-tailed *P* value of < 0.0001. Also, the association between the mycological criteria and panfungal PCR results as assessed by Fischer's exact test is statistically significant with a two-tailed *P* value of 0.0002.

Most of the published studies report the detection of single fungal species or few *Candida* or *Aspergillus* species in the blood samples of patients with IFIs.[4,20] Species- or genus-specific PCR assays only target a narrow spectrum of pathogens, and therefore can only be used if evidence for the infection with a certain pathogen already is present.[3] Panfungal real-time PCR on the other hand enables the unspecific detection and quantification of all fungal DNA present in a sample through the use of universal fungal primers.[21]

The senstivity and specificity values are encouraging and the Panfungal PCR positivity precedes the diagnosis by culture and histopathology by 5 to 8 days as seen in several studies. Delay in the identification of a fungal infection and specific species often enables the infection to progress to a point where subsequent antifungal therapy becomes less effective. The relation between early diagnosis and treatment and mortality was illustrated in a multi-institutional study by Garey *et al.* of 230 patients with candidemia.[22] The results showed a significant trend for increased mortality with progressive delays in the initiation of fluconazole therapy. Patient mortality rates were associated with empiric therapy or zero delay (treatment initiation within 24 hours of time of blood sample collection for culture, but before organism identification) and delays of 1, 2, and ≥3 days were 15%, 24%, 37%, and 41%, respectively. Similarly, von Eiff *et al.*, observed a mortality rate of 90% for patients with pulmonary aspergillosis when antifungal therapy was initiated >10 days after the onset of pneumonia.[23]

Chamilos *et al.* demonstrated that in 60% of histopathology-proven IFI, fungal culture was negative and that histopathology cannot identify fungus genus or species.[24] Therefore, rapid and sensitive

fungal PCR may save precious time in immunocompromised patients with IFI who are already at high risk for severe complications and can direct the appropriate use of early and pre-emptive antifungal therapy.

One of the limitations of our study is that a limited number of patients were included. The incidence of IFIs in our study is much higher than those reported by previous investigators. Panfungal PCR was done only once at the onset of fever. Serial sampling of blood to look for sequential PCR positivity may have had a higher significance. Histopathological diagnosis could not be achieved in any of the cases included in the study due to the thrombocytopenia in almost all of the patients.

To summarise, the sensitivity, specificity, PPV and NPV of panfungal PCR for the diagnosis of invasive fungal infections as compared to the EORTC MSG criteria were 82.76%, 90.48%, 92.31%, and 77.78%, respectively.

In conclusion, panfungal PCR is a promising and highly sensitive diagnostic test for screening of at-risk patients suspected to have IFI. The use of panfungal PCR assay in combination with other diagnostic modalities and clinical judgment can be very helpful, especially in a country like India where the resources are limited and early diagnosis by panfungal PCR positivity at the level of primary healthcare physicians and family physicians can improve the prognosis of patients by early institution of antifungal therapy. It will also help in implementing strategies to reduce the high mortality and morbidity associated with IFI. Furthermore, a negative PCR result can also curtail the usage of unwarranted antifungal therapy in febrile neutropenic patients, especially in resource-poor settings like India.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form, the patients have given their consent for their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Conflicts of interest

There are no conflicts of interest.

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Annexure 1

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) Consensus Group definitions for invasive fungal disease (2008)

Table 1: Criteria for proven invasive fungal disease except for endemic mycoses		
Analysis and specimen	**Molds[a]**	**Yeasts[a]**
Microscopic analysis: sterile material	Histopathologic, cytopathologic or direct microscopic examination[b] of a specimen obtained by needle aspiration or biopsy in which hyphae or melanized yeast-like forms are seen accompanied by evidence of associated tissue damage	Histopathologic, cytopathologic or direct microscopic examination[b] of a specimen obtained by needle aspiration or biopsy from a normally sterile site (other than mucous membranes) showing yeast cells-for example, Cryptococcus species indicated by encapsulated budding yeasts or Candida species showing pseudohyphae or true hyphae[c]
Culture Sterile material	Recovery of a mold or 'black yeast' by culture of a specimen obtained by a sterile procedure from a normally sterile and clinically or radiologically abnormal site consistent with an infectious disease process, excluding bronchoalveolar lavage fluid, a cranial sinus cavity specimen and urine	Recovery of a yeast by culture of a sample obtained by a sterile procedure (including a freshly placed [less than 24 h ago drain) from a normally sterile set showing a clinical or radiological abnormality consistent with an infectious process.
Blood	Blood culture that yields a mold[d] (e.g. *Fusarium* species) in the context of a compatible infectious disease process	Blood culture that yields yeast (e.g. *Cryptococcus* or *Candida* species) or yeastlike fungi (e.g. *Trichosporon* species)
Serological analysis: CSF	Not applicable	Cryptococcal antigen in CSF indicates disseminated cryptococcosis

[a]If culture is available, append the identification at the genus or species level from the culture results. [b]Tissue and cells submitted for histopathologic or cytopathologic studies should be stained by Grocott-Gomorri methenamine silver stain or by periodic acid Schiff stain, to facilitate inspection of fungal structures. Whenever possible, wet mounts of specimens from foci related to invasive fungal disease should be stained with a fluorescent dye (e.g calcofluor or blankophor). [c]Candida, Trichosporon, and yeast-like Geotrichum species and Blastoschizomyces capitatus may also form pseudohyphae or true hyphae. [d]Recovery of Aspergillus species from blood cultures invariably represents contamination.

Table 2: Criteria for probable invasive fungal disease except for endemic mycoses

Host Factors[a]

  Recent history of neutropenia (10 days) temporally related to the onset of fungal disease

  Receipt of an allogeneic stem cell transplant

  Prolonged use of corticosteroids (excluding among patients with allergic bronchopulmonary aspergillosis) at a mean minimum dose of 0.3 mg/kg/day of prednisone equivalent for >3 weeks

  Treatment with other recognized T cell immunosuppressants, such as cyclosporine, TNF-α blockers, specific monoclonal antibodies (such as alemtuzumab), or nucleoside analogues during the past 90 days

  Inherited severe immunodeficiency (such as chronic granulomatous disease or severe combined immunodeficiency)

Clinical criteria[b]

  Lower respiratory tract fungal disease[c]

  The presence of one of the following three signs on CT:

    Dense, well-circumscribed lesions(s) with or without a halo sign

    Air-crescent sign

    Cavity

  Tracheobronchitis

    Tracheobronchial ulceration, nodule, pseudomembrane, plaque or eschar seen on bronchoscopic analysis

  Sinonasal infection

    Imaging showing sinusitis plus at least one of the following three signs:

      Acute localized pain (including pain radiating to the eye)

      Nasal ulcer with black eschar

      Extension from the paranasal sinus across bony barriers, including into the orbit

  CNS infection

    One of the following two signs:

      Focal lesions on imaging

      Meningeal enhancement on MRI or CT

Disseminated candidiasis[d]

  At least one of the following two entities after an episode of candidemia within the previous two weeks:

    Small, target-like abscesses (bull's-eye lesions) in liver or spleen

    Progressive retinal exudates on ophthalmologic examination

Mycological criteria

  Direct test (cytology, direct microscopy or culture)

    Mold in sputum, bronchoalveolar lavage fluid, bronchial brush or sinus aspirate samples, indicated by one of the following:

    Presence of fungal elements indicating a mold

    Recovery by culture of a mold (e.g. *Aspergillus, Fusarium, Zygomycetes* or Scedosporium species)

  Indirect tests (detection of antigen or cell-wall constituents)

  Aspergillosis

    Galactomannan antigen detected in plasma, serum, bronchoalveolar lavage fluid or CSF

  Invasive fungal disease other than cryptococcosis and zygomycoses

    β-D-glucan detected in serum

NOTE. Probable IFD requires the presence of a host factor, a clinical criterion, and a mycological criterion. Cases that meet the criteria for a host factor and a clinical criterion but for which mycological criteria are absent are considered possible IFD. [a]Host factors are not synonymous with risk factors and are characteristics by which individuals predisposed to invasive fungal diseases can be recognized. They are intended primarily to apply to patients given treatment for malignant disease and to recipients of allogeneic hematopoietic stem cell and solid organ transplants. These host factors are also applicable to patients who receive corticosteroids and other T cell suppressants as well as to patients with primary immunodeficiencies. [b]Must be consistent with the mycological findings, if any, and must be temporally related to current episode. [c]Every reasonable attempt should be made to exclude an alternative aetiology. [d]The presence of signs and symptoms consistent with sepsis syndrome indicates acute disseminated disease, whereas their absence denotes chronic disseminated disease. [e]These tests are primarily applicable to aspergillosis and candidiasis and are not useful in diagnosing infections due to *Cryptococcus species* or *Zygomycetes* (e.g., Rhizopus, Mucor, or Absidia species). Detection of nucleic acid is not included, because there are as yet no validated or standardized methods.