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Original Article

Speciation of fungi using real time PCR with molecular beacons: Can we solve the enigma of diagnosis of invasive fungal disease?

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ABSTRACT

Background: Invasive fungal diseases (IFDs) are difficult to diagnose and associated with high mortality rates, especially in the immunosuppressed. Species of *Aspergillus* and *Candida* are the cause of majority of invasive fungal disease however IFDs are also caused by *Fusarium*, *Zygomycetes*, *Trichosporon*, etc. Early detection is crucial for appropriate antifungal therapy. Blood cultures usually fail to isolate filamentous fungi, while detection of circulating beta-D-glucan or galactomannan antigens show variable sensitivity and specificity. There is a need of reliable, sensitive and specific diagnostic tests for IFDs.

Methods: A real-time Polymerase Chain Reaction (PCR) assay with a universal primer/molecular beacon system was developed for detecting and speciating most of the pathogenic fungi implicated in IFD. A single-reaction assay was designed targeting a carefully selected region of the ITS2 and ITS5 subunits of the fungal rDNA gene along with four molecular beacons capable of differential hybridization to the amplicons of different species. This generated a signature set of melting temperatures using the standard strains. The assay was tested on clinical specimens from patients with suspected invasive fungal disease.

Results: The assay was tested on 72 clinical samples and 72 healthy controls. Of these, 22 clinical samples (6/8 proven; 13/29 probable; 3/35 possible IFD, classified by the EORTC/MSG criteria) were positive by PCR and generated a set of melting temperatures enabling identification of the causative fungus. The assay was negative in all healthy controls.

Conclusion: The molecular beacon assay is a promising tool providing a rapid method for detection and monitoring of invasive fungal disease in immunosuppressed patients.

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Introduction

Invasive fungal diseases (IFDs) are a cause of significant morbidity and mortality in immunosuppressed patients. They are relatively common yet pose a diagnostic challenge to the physician. In 90% of all invasive fungal infections in immunocompromised individuals *Candida* and *Aspergillus* species are the causative agents, while remaining are *Fusarium*, *Zygomycetes*, *Trichosporon* spp. and other fungi.^{1–5} Conventional phenotypic identification methods like blood cultures and serological fungal antigen detection assays (e.g. beta-D-glucan or galactomannan) have shown variable sensitivity and specificity.^{6,7} Histopathological examination of biopsies (computed tomography-guided) are quite sensitive and specific, but may give rise to complications.^{8,9} In order to overcome the difficulty in diagnosis, the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group (EORTC) and the Mycoses Study Group (MSG) of the National Institute of Allergy and Infectious Diseases (USA) collectively known as the EORTC/MSG proposed a set of criteria for diagnosis of IFDs in patients of Haemopoietic Stem Cell Transplant (HSCT) and malignancy in 2002 which were further revised in 2008.^{10,11} As per these criteria IFDs can be divided into the three following categories: proven, probable and possible. This is based on a combination of the clinical criteria (including clinical signs and symptoms and radiologic evidence in the form of typical halo or air crescent sign on a chest radiograph), host factors such as neutropenia, steroids or other immunosuppressants and microbiological (culture)/histopathological/serological (antigen detection) evidence of fungal infection. Over the years, there is a felt need for a diagnostic method which would be reliable, sensitive and minimally invasive for diagnosis of IFDs. Non-culture based methods such as antigen detection assays and Polymerase Chain Reaction (PCR) based methods have been developed and have been shown to be fairly useful in diagnosis of IFD.¹² PCR based methods though far from perfect compounded by the presence of low levels of fungal DNA in clinical specimens, are restricted in use owing to limited availability, but show promise as a sensitive and rapid molecular fungal detection method.^{13–16} Increasing incidence of infections have been attributed to yeasts other than *Candida albicans*, *Aspergillus* spp. other than *A. fumigatus* and other opportunistic fungi.^{17,18} A diagnostic PCR assay capable of detecting many uncommon fungal species is required. This can be achieved by a broad-range PCR assay targeting the highly conserved regions of the 5S-18S ribosomal DNA gene cluster and the Inter-Transcribed Spacer (ITS) region to amplify targets from all fungal DNA.¹⁹ Based on this, assays for detection of IFDs using PCR have been developed, however these assays permit detection of limited number of fungal species only or are laborious and time consuming like the restriction fragment length polymorphism (RFLP) assay, PCR sequencing or the DNA micro array based method.^{20–23} Studies using real time hybridization probe based technology have showcased the technique of detection of both filamentous fungi (e.g. *Aspergillus* spp.) and yeasts (e.g. *Candida* spp.) in a single well assay.^{13,24} The importance of speciation of fungi by a molecular detection method is underscored by the fact that

some of the fungi exhibit intrinsic resistance to various groups of antifungals: e.g. *Candida glabrata* and *Candida krusei* show intrinsic resistance to fluconazole and itraconazole, while *Candida lusitanae* and *Aspergillus terreus* exhibit intrinsic resistance to amphotericin B and *Cryptococcus* species are resistant to echinocandins.^{25–27}

A real-time PCR fungal detection assay is described here which was developed to attempt speciation of most of the pathogenic fungi implicated in IFD. Panfungal primers chosen were capable of amplifying the 18S rDNA region targeting a carefully selected segment of the ITS 2 and ITS 5 subunit.¹⁹ Molecular beacons were used for detection of the amplicons.²⁸ Molecular beacons are oligonucleotide probes which have complementary sequences at the ends that result in a hairpin conformation in the unhybridized state. The ends are labelled with a fluorescent reporter dye and a quencher molecule. In the unhybridized state the proximity of the reporter and the quencher prevents any fluorescence. The loop portion of the beacon serves as the probe which on annealing to the target nucleic acid separates the fluorophore and the quencher thereby permitting the fluorescence of the reporter dye (Fig. 1). Here the probe–target hybrid is longer and more stable than the stem of the probe. A point to note here is that the beacon does not get hydrolysed in this process as compared to a hydrolysis (Taqman) probe.²⁸ Molecular beacons designed for different target sequences and labeled with differently colored fluorophores can be used for identification of multiple targets in the same assay tube.²⁹ The molecular beacon sequences can be further modified to allow differential hybridization with similar targets differing by a few nucleotides. This property of differential hybridization has been exploited, targeting the 16S rDNA regions of bacterial genomes in the pioneering work by Chakravorty et al.³⁰ When subjected to denaturation the target/beacon hybrids melt apart at different temperatures depending on the difference in nucleotide sequence between different targets. This enables creation of a signature set of melting temperatures (T_m) when multiple beacons are used. In the present study, we have designed a set of molecular beacons targeting the ITS subunit in the 18S-rDNA region of the fungal genome.

Materials and methods

The study was carried out in the Department of Microbiology of a Medical College over a period of one and a half years as a diagnostic test evaluation study. The sample size was calculated for estimating the sensitivity of a new test. Taking the projected sensitivity of the new test as 90% with a precision of 0.07 and a confidence interval of 95% the sample size worked out to 71 along with an equal number of controls. During the study period 72 cases could be collected. Blood specimens were also processed from 72 healthy controls from among the health care staff of our institution.

DNA extraction

Initially 20 fungal strains comprising both American Type Culture Collection (ATCC) and laboratory confirmed clinical

Molecular beacons

Fig. 1 – The molecular beacon is designed to have sequences at the ends of the probe that create a complementary stem, resulting in a shape similar to a hair pin. On annealing to the target the stem separates and results in real time fluorescence as the quencher moves away from the reporter dye.

Fig. 2 – Representative gel image of PCR amplicons generated by various fungal species using universal primers ITS5 and ITS2. Molecular beacons were designed. After capillary sequencing of the amplicons. Crypto – *Cryptococcus neoformans*, C.trop – *Candida tropicalis*, C.para – *Candida parapsilosis*, MM – 100 bp DNA ladder, A. flu – *Aspergillus flavus*, Rhizopus – *Rhizopus* spp., NC – negative control.

isolates were subjected to the assay. The fungal strains were sub-cultured on Sabouraud dextrose agar and then suspensions were prepared in sterile phosphate buffer saline. The DNA extraction procedures for the fungi were carried out in a Class 2 Biosafety cabinet in a dedicated room employing suitable biosafety precautions and appropriate Bio-waste disposal methods. Fungi/Yeast Genomic DNA Isolation Kit (Norgen Biotek Corp, Canada) with modifications in the form of lyticase and proteinase K (Sigma, India) digestion was used for extraction of DNA from both fungal culture and clinical specimens. Bacterial nucleic acid was extracted using an automated nucleic acid extraction system (EasyMag, BioMerieux, France). The eluted DNA was stored at -20°C till further

processing. All reagents used in the processing and further amplification were certified mycology grade.

Target sequence analysis and molecular beacon design

Panfungal primers capable of amplifying the 18S rDNA region targeting a carefully selected segment of the ITS 2 and ITS 5 subunit were chosen.¹⁹ Conventional PCR was carried out to amplify the fungal DNA (Fig. 2) in a total reaction volume of 25 μL comprising 12.5 μL of PCR master mix (ThermoFisher, USA), 0.2 pmol of forward and reverse primers and 5 μL of DNA template. Target DNA was amplified in a GeneAmp Thermal Cycler (Perkin Elmer, USA). Cycling conditions were set at

initial denaturation for one cycle at 95 °C for 3 min and then 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 45 s and a final extension step at 72 °C for 10 min. The products of the PCR were then run on a 1.5% agarose gel to demonstrate the amplicons generated by the various fungal strains. A 100 bp molecular marker (HiMedia India) was run in parallel in order to determine the approximate sizes of the amplicons. An image of a representative gel electrophoresis of the amplified products is shown in Fig. 2. Amplicons generated from 11 standard/laboratory confirmed fungal strains including *Candida albicans*, *Candida tropicalis*, *Candida tropicalis*, *C. krusei*, *Candida parapsilosis*, *Cryptococcus neoformans*, *Trichosporon asahii*, *A. fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, *Mucor* spp., *Rhizopus* spp. were sequenced by the capillary sequencing method (Eurofins Genomics India Pvt Ltd) using the same primers which had been used for initial amplification. The sequence data so obtained were then aligned and matched with the genomic database, in order to confirm the molecular identity of the fungus, using the Basic Local Alignment Search Tool (BLAST) available on the website of the National Centre for Biotechnology Information (NCBI).³¹

These sequences after confirmation were aligned using MEGA version 6 gene alignment software and four molecular beacons were designed by us using the DNA fold programme available on the website of the University of Albany, USA (<http://unafold.rna.albany.edu/?q=mfold/dna-folding-form>) (Fig. 3) taking the constant and hypervariable regions into consideration.^{32,33} The four molecular beacons were capable of differential hybridization to the amplicons of different species and this property was exploited to generate melting curves and a signature set of melting temperatures (T_m) using the standard strains.²⁸

The beacons were labelled at the 5' end with four different dyes (FAM, TAMRA, Texas Red, Cy5) and at the 3' end with quenching dyes (BHQ1 or BHQ2) and named as panfungal with *C. albicans* as base sequence (PFC11), panfungal with *A. fumigatus* as the base sequence (PFA21), pan-zygomycetes with *Mucor* as base sequence (PFM3) and pan-yeast with *C. tropicalis* as base sequence (PYCT41). Sequences of the primers and molecular beacons (Eurofins Genomics India Pvt Ltd, Bangalore, India) are given in Table 1.

The molecular beacon assay for identification of various fungi by real time PCR was carried out on a Light Cycler 480 II instrument (Roche Diagnostics, USA). Each reaction was setup in a volume of 20 µL. The amplification was performed using the same parameters as above in a 96 well PCR plate and each reaction mixture comprised sample DNA extracted from the fungal cultures, a Real Time PCR master mix 5X Real Time Light Cycler Genotyping Master (Roche Diagnostics India) and the set of molecular beacons (Eurofins Genomics India Pvt Ltd). Post amplification melt curve analysis was carried out with initial heating to 95 °C, cooling to 35 °C and gradual heating again to 95 °C with temperature recording set at 10 acquisitions per degree rise in temperature. The temperatures were recorded using T_m calling software in the Roche Light Cycler instrument (Fig. 4). In addition, DNA template from a panel of bacteria comprising *Streptococcus* spp., *Staphylococcus* spp., *Pseudomonas aeruginosa*, *Enterococcus faecalis*, and *Mycobacterium tuberculosis* was also subjected to the assay for checking cross reactivity of the primers and probes. A set of melting

temperatures (T_m) generated by the standard fungal strains were used to create a reference set of melting temperature (T_m) values using the four beacons (Table 2).

After standardization with standard fungal strains, the assay was tested on clinical specimens from patients with suspected fungal infections. A total of 72 patients with suspected invasive fungal disease characterized as per the EORTC/MSG criteria into 8 proven, 29 probable, 35 possible, were tested.¹¹ The specimens included peripheral blood, bronchoalveolar lavage (BAL) and ascitic fluid. The blood samples were inoculated into blood culture bottles and incubated in an automated system with an extended protocol of 14 days (BACTEC 9120, BD USA) while the BAL and body fluids were inoculated onto Sabouraud's dextrose agar. Blood samples from an equal number of healthy volunteers were tested as controls to check for false positivity.

The identification of the fungus detected from the clinical samples by the beacon assay was done by matching the T_m values generated from the sample compared with the set of reference values generated as above.

Results

Conventional PCR carried out on the standard strains gave different amplicons of different base pair sizes as depicted in Fig. 1, which were sequenced and then the molecular beacons were synthesized for the real time assay.

The real time PCR incorporating a melt curve analysis resulted in generation of different melting temperatures with respect to each beacon for each standard fungal strain tested. The melting curves and T_m °C generated by the four Beacons from the standard strains are depicted in Fig. 4. The reference set of melting temperatures (T_m) in °C generated for the 20 standard/clinical fungal strains using the four molecular beacons is shown in Table 2. For example beacons PFC11, PFA21 and PYCT41 generated T_m of 64.26, 70.38 and 80.23 °C respectively with *A. flavus* (row 1 of Table 2), while T_m generated by the same beacons with *Aspergillus fumigatus* were 69.59, 74 and 64.93. It is apparent that the signature set of T_m differs between the two species of *Aspergillus*. The same set of beacons gave similarly varying T_m which could help differentiate the yeasts (*Candida*, *Cryptococcus*, *Trichosporon*, etc.) too. Here it was found that only the zygomycetes gave a T_m with all the four beacons including PFM3. The other fungi did not generate any T_m with Beacon PFM3. Beacon PYCT41 gave T_m with adequate differences amongst the yeasts, thus helping in speciation of yeasts. However, *C. krusei* and *Candida guilliermondii* could not be differentiated as both these did not generate any T_m with this beacon.

Real time PCR was performed on 72 clinical samples and on blood samples from 72 healthy controls. Of the 72 clinical samples 22 (6/8 proven; 13/29 probable; 3/35 possible) were positive by PCR with generation of different melting temperatures whereas the PCR was negative in all healthy controls.

The identification chart of positive samples is as given in Table 3. The T_m set generated by the clinical samples was matched with the set created from the standard strains thereby helping in identification of the fungus. No cross reactivity of the molecular beacons between fungi and

Fig. 3 – Structure of the four molecular beacons: panels a and b show the panfungal beacons (PFC11 and PFA21), panel c depicts Pan zygomycetes beacon (PFM3), panel d shows pan-yeast beacon (PYCT41).

bacterial pathogens was observed. When compared with culture, sensitivity of the PCR assay was 75%, and specificity was 76.6%. The positive predictive value (PPV), negative predictive value (NPV) worked out to be 28.6%, 96.1% respectively.

Discussion

The clinical diagnosis of IFD is inherently difficult and this led to the formulation of diagnostic criteria by the EORTC/MSG for

Table 1 – Nucleotide sequences of primers and molecular beacons used in the assay. The stem of the molecular beacons are shown in lower case. The beacons are labelled at the 5' and 3' prime ends with the fluorescent dye and the quencher respectively. 6FAM: 6-carboxyfluorescein, TAMRA: 5-carboxytetramethylrhodamine, Texas Red: sulforhodamine, Cy5: cyanine, BHQ1: black hole quencher1; BHQ2: black hole quencher2.

Primer/beacon name	Primer/probe sequence 5'–3'	5'/3' fluorescent dye/quencher label	Reference
ITS 5	GGAAGTAAAGTCGTAACAAGG	–	White et al. ²⁰
ITS 2	GCTGCGTTCTTCATCGATGC	–	White et al. ²⁰
PFC11	gcgccAAAACTTTCAACAACGGATCTCTTGGTTCCGGCATggcgc	6FAM/BHQ1	This study
PFA21	cgcgTGACAGCGCGGATGCTAGATGTCATTACCGAGTGAGacgcg	TAMRA/BHQ2	This study
PFM3	gcgcgcTCGATCCAGGGCTCGTACCTTAGGGTTTCTCTGGGgcccgc	TEXASRed/BHQ2	This study
PYCT41	gccggCAAACCTTGATTATTATTACAATAGTCAAACTTTcgggc	Cy5/BHQ2	This study

Fig. 4 – Melt curves and temperature of melting (T_m °C) peaks generated by the four beacons in a real time thermal cycler (Roche LC480 II) after amplification of DNA from various fungal strains. The top of each panel shows the meting curves while the bottom half shows the melting peaks. panel a: beacon PFC11, panel b: beacon PFA21, panel c: beacon PFM3 (zygomycetes), panel d: PYCT41 (yeast).

the diagnosis of IFD.^{10,11} However, even within the criteria only the category of proven IFD with a positive microbiological/histopathological result can be considered a robust diagnosis.¹¹ These investigations require invasive procedures for collection of appropriate samples. This highlights the need of minimal/non-invasive, rapid diagnostic methods, of which the polymerase chain reaction is a promising method.⁷

Various workers have utilized the PCR for detection of IFD. However many of them have used conventional PCR followed by post amplification processing involving RFLP or sequencing which are labour intensive and have a long turnaround time.^{12,20} Others brought in real time PCR as a better alternative to conventional PCR.^{12,13,20,34,35} Sensitivity of real-time PCR is better than conventional PCR, Secondly, real-time PCR has a better turnaround time as compared to conventional PCR because the post amplification processing is eliminated. Thirdly, owing to a combination of target amplification and detection in a single, closed-reaction vessel,

real-time PCR obviates the possibility of carry over contamination by amplicons. However studies involving real time PCR for diagnosis of IFD target only major fungal genera without speciation or achieve limited speciation.¹⁵ The aim of our study was the development and evaluation of a real-time panfungal PCR with molecular beacons. An assay was designed involving a universal primer/molecular beacon system for both detection and molecular speciation of fungi.

The sensitivity and specificity of our assay as compared to culture worked out to 75% and 76.6% respectively, in addition to determining the species of the fungi causing the IFD. Jordanides et al. showed a sensitivity of 75% and a specificity of 70% when evaluating a real time PCR assay wherein two hybridization probes were used targeting the 18s rDNA segment of the fungal genome which however could only detect two broad groups of fungi: *Aspergillus* group or *Candida* group.¹³ Similar sensitivity and 92% specificity was shown by El-Mahallawy et al. however, again the PCR did not differenti-

Table 2 – Reference set of melting temperatures (T_m °C) generated using the four molecular beacons following amplification of DNA from standard fungal strains. Beacon PFC11 gave a T_m with all strains tested while PFA21 and PYCT41 gave adequate difference in T_m to enable speciation. Beacon PFM3 gave T_m with only zygomycetes.

ATCC no.	Fungal strains	Molecular beacon			
		PFC11	PFA21	PFM3	PYCT41
ATCC 204304	<i>Aspergillus flavus</i>	64.26	70.38	–	82.23
ATCC 293	<i>Aspergillus fumigatus</i>	69.59	74	–	64.93
ATCC 9508	<i>Aspergillus niger</i>	64.17	69.92	–	64.93
Clinical isolate	<i>Aspergillus terreus</i>	64.12	70.47	–	82.8
Clinical isolate	<i>Aspergillus nidulans</i>	64.21	70.34	–	81.96
Clinical isolate	<i>Aspergillus glaucus</i>	63.75	70.2	–	46
Clinical isolate	<i>Mucor</i>	49.66	46.44	59.03	61.72
Clinical isolate	<i>Rhizopus</i>	63.9	46.61	58.59	61.49
ATCC 90028	<i>Candida albicans</i>	65.19	70.34	–	45.63
ATCC 750	<i>Candida tropicalis</i>	65.19	70.31	–	62.47
ATCC 6258	<i>Candida krusei</i>	65.33	70.38	–	–
ATCC 22019	<i>Candida parapsilosis</i>	65.23	70.31	–	41.19
ATCC 6258	<i>Candida guilliermondii</i>	64.75	70.25	–	–
ATCC 208821	<i>Cryptococcus neoformans</i>	62	68.91	–	61
Clinical isolate	<i>Trichosporon asahii</i>	61.09	69.04	–	61.72
Clinical isolate	<i>Penicillium</i> spp.	68.5	73.17	–	–
Clinical isolate	<i>Fusarium</i> spp.	55.1	57.22	–	–
Clinical isolate	<i>Microsporum gypseum</i>	68.35	73.08	–	–
Clinical isolate	<i>Epidermophyton mentagrophytes</i>	63.2	–	–	–
Clinical isolate	<i>Trichophyton rubrum</i>	61.58	–	–	–

Table 3 – Melting temperatures (T_m °C) of the beacons generated after PCR on clinical samples. The fungus was identified by matching these with the reference set of T_m in Table 2.

Sample no.	PFC11	PFA21	PFM3	PYCT41	Fungus identified
1	64.62	70.25	–	–	<i>Candida guilliermondii</i>
7	64.75	70.25	–	–	<i>Candida guilliermondii</i>
12	63.92	70.2	–	46	<i>Aspergillus glaucus</i>
14	68.96	73.17	–	–	<i>Penicillium</i> spp.
17	68.59	73.27	–	–	<i>Penicillium</i> spp.
19	64.37	70.38	–	82.23	<i>Aspergillus flavus</i>
21	64.98	70.25	–	–	<i>Candida krusei/guilliermondii</i>
24	64	70.38	–	82.23	<i>Aspergillus flavus</i>
25	65.8	70.38	–	–	<i>Candida krusei/guilliermondii</i>
26	64.27	70.38	–	82.23	<i>Aspergillus flavus</i>
29	64.18	69.9	–	64.95	<i>Aspergillus niger</i>
30	64.17	69.92	–	64.93	<i>Aspergillus niger</i>
31	60.8	69.04	–	61.72	<i>Trichosporon asahii</i>
33	60.9	69.1	–	61.7	<i>Trichosporon asahii</i>
41	69.59	74	–	64.93	<i>Aspergillus fumigatus</i>
47	65.19	70.34	–	45.63	<i>Candida albicans</i>
49	64.98	70.34	–	45.62	<i>Candida albicans</i>
52	55.1	57.22	–	–	<i>Fusarium</i> spp.
55	69.3	74	–	64.93	<i>Aspergillus fumigatus</i>
56	64.27	70.38	–	82.23	<i>Aspergillus flavus</i>
67	65.06	70.54	–	41.2	<i>Candida parapsilosis</i>
68	65.2	70.34	–	62.47	<i>Candida tropicalis</i>

ate the species of the fungi.¹⁴ The study by Spiess et al.²³ achieved a similar sensitivity while employing a technically demanding DNA microarray technique. In another study by Lass-Flörl et al. a broad-range PCR done for IFD showed a sensitivity, specificity, positive predictive value, and negative predictive value of 57.1%, 97.0%, 80%, and 91.7%, respectively, for microscopy-negative fungal infections.¹⁵ Here the authors compared microscopy with PCR from samples other than blood and this is the reason for the higher PPV obtained in their

study.¹⁵ The specificity is low our study because the gold standard, i.e. culture failed to grow filamentous fungi (e.g. *Aspergillus* spp.) and the only specimens that were processed were blood cultures and body fluids. Valero et al. have worked on similar lines as the present study however majority of their samples were from biopsies which would explain their higher sensitivity of 83.3% as well as higher culture positivity.³⁶ We found that the blood culture bottles grew only yeasts and not the filamentous fungi. This is supported by other studies that

have demonstrated that automated blood culture bottles support the growth of yeast (e.g. *Candida* spp.) better as compared to filamentous fungi (e.g. *Aspergillus* spp.).⁶ Two samples from among the proven cases that were positive in culture turned out to be negative in PCR. Both these samples grew non-albicans *Candida* on culture. The negative PCR in these cases could have been owing to factors like low level of candidemia/nucleic acids in the sample.

The assay developed in our study has an advantage that it can identify the species of fungus involved. Intrinsic resistance exhibited by some fungi to some of the antifungals underlines the requirement of speciation of fungi so that appropriate therapy can be administered in a timely fashion.^{25–27} As far as turnaround time is concerned, the time taken for the PCR based detection is only 7–8 h whereas culture takes 24–48 h for culture isolation of yeasts. Similarly, in the case of filamentous fungi culture takes more than 7 days and also prove difficult to culture from peripheral blood specimens.⁶ Screening of patients who are at risk of invasive fungal disease using a sensitive diagnostic test, will be the focus of therapeutic strategies to reduce the incidence of IFD and the associated mortality. Molecular beacons with differential hybridization to fungal targets will be of help in early detection and speciation of the fungal organism and enable wide range detection of fungi making it an ideal candidate for a rapid diagnostic test relative to antigen detection or culture. Speciation of fungi with molecular beacons presents a promising molecular method for the screening and monitoring of invasive fungal infection, which can be readily applied to clinical situations.

Conflicts of interest

The authors have none to declare.

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