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Dual Panel Multiplex PCR Assay for Rapid Detection of Medically Important Fungi and Resistant Species of *Candida* and *Aspergillus*

(Asai PCR Multipleks Dual Panel untuk Pengesanan Segera Kulat yang Penting daripada Segi Perubatan dan Spesies Rintang *Candida* dan *Aspergillus*)

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ABSTRACT

Invasive fungal infections (IFIs) have risen dramatically in recent years among high risk immunocompromised patients. Rapid detection of fungal pathogens is crucial to timely and accurate antifungal therapy. Two multiplex polymerase chain reaction (PCR) assays were developed to detect major fungal species that cause invasive infections and identify resistant species. Genus specific primers for Candida, Aspergillus, Fusarium *and species specific primers for* Candida glabrata, Candida krusei *and* Aspergillus terreus *which are known to be clinically resistant species, were designed from the internal transcribed spacer (ITS) regions of ribosomal ribonucleic acid (rRNA) gene complex. Both assays were performed simultaneously to promote rapid detection of fungal isolates based on distinct amplicon sizes. Inclusion of the universal fungal primers ITS 1 and ITS 4 in the genus specific assay produced a second amplicon for each isolate which served to confirm the detection of a fungal target. The limit of detection for the genus specific assay was 1 nanogram (ng) deoxyribonucleic acid (DNA) for* Aspergillus fumigatus *and* Candida albicans, *0.1 ng DNA for* Fusarium solani, *while the species-specific assay detected 0.1 ng DNA of* A. terreus *and 10 picogram (pg) DNA of* C. krusei *and* C. glabrata. *The multiplex PCR assays, apart from universal detection of any fungal target, are able to detect clinically important fungi and differentiate resistant species rapidly and accurately, which can contribute to timely implementation of effective antifungal regime.*

Keywords: Aspergillus*;* Candida*; detection;* Fusarium*; multiplex PCR*

ABSTRAK

Jangkitan kulat invasif telah meningkat sejak kebelakangan ini dalam kalangan pesakit terimunokompromi. Pengesanan segera patogen kulat adalah amat perlu supaya terapi anti kulat yang bersesuaian dapat diberikan. Dua asai tindak balas rantai polimerase multipleks telah dibangunkan untuk mengesaan spesies utama patogen kulat yang menyebabkan infeksi invasif dan mengenal pasti spesies resistan. Primer khusus untuk genus Candida, Aspergillus, Fusarium *dan khusus untuk spesies* Candida glabrata, Candida krusei *dan* Aspergillus terreus *yang merupakan spesies rintang telah direka cipta berdasarkan jujukan mentranskripsi jarak dalaman (ITS) kompleks gen rRNA. Kedua-dua asai dijalankan serentak untuk mempercepatkan pengesanan pencilan kulat berdasarkan saiz amplikon yang terhasil. Dalam asai khusus untuk pengesanan genus, primer universal kulat disertakan bersama supaya amplikon kedua terhasil bagi setiap pencilan yang mengesahkan kehadiran kulat. Tahap pengesanan untuk asai khusus genus adalah 1 nanogram (ng) asid deoksiribonukleik (DNA)* Aspergillus fumigatus *dan* Candida albicans *serta 0.1 ng DNA* Fusarium solani, *manakala asai khusus spesies dapat mengesan 0.1 ng DNA* A. terreus *dan 10 pikogram (pg) DNA* C. krusei *serta* C. glabrata. *Selain daripada pengesanan semua kulat secara am, asai tindak balas rantai polimerase multipleks yang dibangunkan dapat mengesan kulat berkepentingan klinikal dan membezakan spesies rintang secara pantas dan tepat, justeru boleh berperanan dalam penentuan awal ubatan anti kulat yang efektif bagi pesakit.*

Kata kunci: Aspergillus*;* Candida*;* Fusarium*; PCR multipleks; pengesanan*

INTRODUCTION

The last two decades has seen a growing number of fungal infections coincident with a dramatic increase in the population of severely immunocompromised patients due to human immunodeficiency virus infections, organ transplants, haematological disorders such as leukaemia and other malignancies (Oren & Paul 2014). Intensive and aggressive medical practices and treatments such as surgery, the use of catheters, injections, radiation, chemotherapy, antibiotics and steroids are risk factors for fungal infections (Galimberti et al. 2012). However, these procedures are necessary and therefore, the incidence of fungal infections are expected to increase.

Although *Candida* and *Aspergillus* species are major contributors to life-threatening infections, occurrence of resistant species and the emergence of previously rare fungal species is increasing, resulting from antifungal prophylaxis in high-risk patients (Preuner & Lion

2009; Rishi & Clark 2011). *Candida albicans* is still the predominant species causing infections, however an upward trend was observed for non-*albicans Candida* infections caused by *C. glabrata*, *C. parapsilosis* and *C. krusei* (Pfaller & Diekema 2007; Rishi & Clark 2011). These *Candida* species show reduced susceptibility towards newer echinocandin drugs such as caspofungin, while *C. glabrata* and *C. krusei* are widely resistant to the long-standing, most frequently used azole drug, fluconazole (Pfaller & Castanheira 2016). Prolonged caspofungin prophylaxis in neutropenic patients has contributed to a significant increase of infections caused by *C. krusei* and *C. glabrata* (Lortholary et al. 2011; Wisplinghoff et al. 2014). *Aspergillus fumigatus* continues to be the most frequent causative agent of invasive aspergillosis however, non-*fumigatus Aspergillus* infections increasingly occurred (Azab et al. 2015; Bašková & Buchta 2012). In particular, *A. terreus* has been recognised as resistant towards amphotericin B, the highly effective but also highly toxic broad-spectrum antifungal drug, with a high mortality rate for invasive infections (Blum et al. 2013; Steinbach & Perfect 2003). Less frequently observed fungal genera associated with very high fatality include *Fusarium*, *Scedosporium* and members of Mucorales, namely *Rhizopus* and *Mucor* (Bašková & Buchta 2012). In patients with haematological malignancies, *Fusarium* is the second or third most common mould causing infections (Tortorano et al. 2014)and are reported to have low susceptibility towards azole drugs (Alastruey-Izquierdo et al. 2008). *Fusarium solani* is the species responsible for most human infections (50%) followed by *F. verticillioides* (20%) and *F. moniliforme* (Dignani & Anaissie 2004).

The standard approaches used for the diagnosis of invasive fungal infections include serological detection of circulating fungal antigens, culture of body fluids for fungal recovery followed by identification and histopathological examination of tissue sections for the presence of fungi. Although serological assays are widely used, each one is designed to detect a single fungal genus and tests for *Aspergillus* and *Candida* have shown variable sensitivity and specificity (Bašková & Buchta 2012; Denning 1998), which is similar to culture diagnosis. While histological analyses of biopsy tissues is highly sensitive and specific (Lenka et al. 2007), the method is frequently associated with bleeding complication in patients with severe thrombocytopenia (Denning 1998). Poor outcome in patients with invasive fungal infections (IFIs) is related to delayed institution of an effective antifungal regime and prescription of unnecessary toxic antifungal agents (Yeo & Wong 2002).

In recent years, nucleic acid detection techniques have been developed to provide an early diagnosis of mycotic infections and the identification of pathogenic fungi. Polymerase chain reaction (PCR) based methods including nested or semi-nested PCR assays are particularly promising because of their simplicity, specificity and sensitivity (Cerikçioğlu et al. 2010; Than et al. 2012). Multiplex PCR has been used to detect *Candida* spp. (Mallus et al. 2013; Vahidnia et al. 2015) and *Aspergillus* spp. (Amini et al. 2015; Logotheti et al. 2009) as has real-time PCR (Emam & Abd El-salam 2015; Horváth et al. 2013). Several PCR techniques have targeted ribosomal DNA of Candida (Cerikçioğlu et al. 2010; Mallus et al. 2013; Than et al. 2012) and Aspergillus (Walsh et al. 2011). Although these PCR methods have been useful for the identification of fungal species, they either only identify species within a particular genus or detect the fungus at genus level. Furthermore, real-time PCR requires the use of costly reagents and instrumentation.

Therefore, in this study multiplex PCR assays were developed for simultaneous detection of *Candida*, *Aspergillus*, *Fusarium* and the antifungal-resistant species *A. terreus*, *C. glabrata* and *C. krusei* through distinct amplicon sizes. An optimized multiplex PCR assay with gel electrophoresis detection is a very useful, low-cost method for the detection of major fungal targets, simultaneously.

Materials and Methods

Primers were designed from internal transcribed spacer (ITS) regions of fungal ribosomal gene complex (Table 1). The rDNA sequences of the fungi were retrieved from GenBank database and were subjected to the software CLUSTALW (http://www.genome.jp/tools/clustalw) for multiple sequence alignment. The sequences were analyzed with BioEdit Sequence Alignment Editor Version 7.2.3 for nonhomologous regions among 101 different fungal strains (Table 2). The conserved regions within each targeted genus and species were compared for sequence consistency against multiple strains of each species. The primer regions were tested in silico using the BLAST program (http://blast.ncbi.nlm.nih.gov) to check for specificity towards the target fungi. Genus/species-specific primer sequences were analyzed in OligoAnalyzer 3.1 program (https://sg.idtdna.com/analyzer/Applications/OligoAnalyzer) to analyse its reverse complementary sequence, melting temperature, GC content and molecular weight. The selected primers were synthesized by IDT Singapore.

A total of 84 clinical fungal isolates were evaluated in this study. These include the filamentous fungi *Aspergillus fumigatus* (*n*= 4), *A. niger* (*n*=9), *A. flavus* (*n*=3), *A. terreus* (*n*=1), *Fusarium solani* (*n*=11) and the yeast *Candida albicans* (*n*= 27), *C. tropicalis* (*n*=8), *C. parapsilosis* (*n*=6), *C. krusei* (*n*=3), *C. glabrata* (*n*=9), *C. kefyr* (*n*= 1), *C. dublinensis* (*n*=1) and *Cryptococcus neoformans* (*n*=1). Additionally, 6 ATCC (American Type Culture Collection) strains of *C. albicans* ATCC 10231, *C. glabrata* ATCC 66032, *C. krusei* ATCC 6258, *C. parapsilosis* ATCC 20246, *A. fumigatus* ATCC 204305 and *F. solani* ATCC 36031 were evaluated. Clinical fungal isolates were provided by the Mycology Unit, UKMMC (Universiti Kebangsaan Malaysia Medical Centre) and were identified using routinely used methods which include biochemical tests for yeasts and microscopic morphology for moulds. In addition, DNA from bacterial isolates namely *Escherichia coli* and *Staphylococcus aureus* was also evaluated for

control purpose. The bacterial isolates were obtained from the culture collection of the Novel Antibiotic Research Laboratory, UKM.

The fungal isolates were sub-cultured onto Potato-Dextrose Agar (PDA) or Sabouraud-Dextrose Agar (SDA) (Difco Laboratories, West Molesey, UK) media at 25-30ºC; 2 days for the yeast and 2 weeks for the moulds. To extract DNA, two hundred microliters of fungal cell suspension was placed on QIAcard FTA Card (Qiagen, Hilden, Germany), allowed to dry and microwaved on high power for 30 s. Two discs of 3.0 mm diameter of QIAcard FTA was punched using Harris Uni-Core™ micro-puncher (Ted Pella, California, USA) followed by extraction with Extract-N-Amp™ Plant PCR kit (Sigma, Missouri, USA). The extracted genomic DNA was diluted ten-fold with nuclease free water before proceeding to PCR amplification (BIO-RAD T 100™

TABLE 1. Genus and species specific primers for fungal targets (patent pending)

Target	Primer designation	Sequence (5'→3')	Approximate amplicon size (base pair)
Universal detection			
All fungi	ITS1	TCC GTA GGT GAA CCT GCG G	500 for *Candida albicans*, *C. dubliniensis*, *C. parapsilosis*, *C. tropicalis, C. kefyr*, 480 for *C. krusei* & 860 for *C. glabrata*
	ITS4	TCC TCC GCT TAT TGA TAT GC	550 for *Aspergillus fumigatus*, *A. niger*, *A. flavus*, *A. terreus* & *Fusarium solani*
Genus-specific detection			
Aspergillus	GSAsp1	AAT CAC ACT CAG ACT GCA	200
Candida	GSCand1	GTA TYR CTC AAY ACC AAA C	340; 600 for *C. glabrata*
Fusarium	GSFus1	TAC TAC GCW ATG GAA GCT	450
Species-specific detection			
Aspergillus terreus	SSAter1	CAA GTT GCA AAT AAA TGC	510
Candida glabrata	SSCglab1	GCA GAT TAA TAG AGA AGC TTG	700
Candida krusei	SSCkrusei2	CTC TGC GCA CGC GCA AGA TG	280

TABLE 2. Strains analysed and experimentally tested for genus specific primer design for *Candida*, *Aspergillus*, *Fusarium* and species specific primer for *Aspergillus terreus*, *Candida glabrata*, *Candida krusei*

Genus (No. of strains analysed *in silico*)	(No. of strains experimentally tested)
Candida spp. (Total = 32)	*Candida* spp. (Total = 59)
C. albicans (4)	*C. albicans* (28)
C. dubliniensis (1)	*C. dubliniensis* (1)
C. tropicalis (2)	*C. tropicalis* (8)
C. parapsilosis (3)	*C. parapsilosis* (7)
C. krusei (11)	*C. krusei* (4)
C. glabrata (10)	*C. glabrata* (10)
C. guilliermondi (1)	*C. kefyr* (1)
Aspergillus spp. (Total = 31)	*Aspergillus* spp. (Total = 18)
A. fumigatus (8)	*A. fumigatus* (5)
A. terreus (9)	*A. terreus* (1)
A. clavatus (3)	
A. niger (6)	*A. niger* (9)
A. flavus (4)	*A. flavus* (3)
A. nidulans (1)	
Fusarium spp. (Total = 12)	*Fusarium* spp. (Total = 12)
F. solani (9)	*F. solani* (12)
F. oxysporum (3)	
Species (No. of strains analysed *in silico*)	(No. of strains experimentally tested)
C. glabrata (9)	*C. glabrata* (3)
C. krusei (9)	*C. krusei* (3)
A. terreus (8)	*A. terreus* (1)

Thermal Cycler, Germany).

The multiplex PCR reaction for fungal genus detection contained 2 µL of genomic DNA, 2× GoTaq® Green Master Mix that consisted of GoTaq® DNA Polymerase, 400 µM dNTP and 3mM $MgCl_2$ (Promega, Madison, WI, USA), 0.1 µM of primer ITS 4 and 0.4 µM of primer ITS 1, genus specific *Aspergillus* (GSAsp1), *Candida* (GSCand1), *F. solani* (GSFus1) and species specific *C. krusei* (SSCkrusei2) primers in a total volume of 10 µL. Multiplex PCR reaction for resistant species identification contained 2 µL of genomic DNA, 2× GoTaq® Green Master Mix and 0.4 µM of ITS 1 primer and species specific *C. krusei* (SSCkrusei2), *C. glabrata* (SSCglab1) and *A. terreus* (SSAter1) primers in a total volume of 10 µL. Optimized PCR amplification conditions were: 5 min initial 94ºC step, followed by 34 cycles at 94ºC for 1.5 min, 51.5ºC for 2 min and 65ºC for 3 min and a final extension step at 65ºC for 10 min. A patent of the designed genus and species specific primers (Table 1) and multiplex PCR assay procedure submitted to the Intellectual Property Corporation of Malaysia (MyIPO) is pending (Malayisa Patent Application No. PI 2015001974). Amplification products were electrophoresed in agarose gels (3.0% w/v) (Vivantis, California, USA) in 0.5 ×TBE buffer and stained with ethidium bromide (Nacalai Tesque, Kyoto, Japan).

The limit of detection of the multiplex PCR assays was determined using serial dilutions of fungal DNA extracted with Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA).

RESULTS

Each genus and species-specific primer was specific for its target as tested in multiplex PCR assays (Figures 1 and 2) and all the 83 fungal targets were correctly identified according to amplicon size. All *Aspergillus* species strains (*n*=17) were successfully amplified by ITS 1- GSAsp1 primer pair producing amplicons of approximately 200 base pair (bp). The *Candida* genus-specific primer pair ITS1-GSCand1 amplified an approximately 340 bp product for all *Candida* spp. Strains (*n*=43) except for *C. glabrata* strains (*n*=9) which had an approximate amplicon size of 600 bp (Figure 3). The *Candida* genus-specific primer did not amplify *C. krusei* strains. Therefore, *C. krusei*-specific primer pair ITS1-SSCkrusei2 was included in the PCR reaction and produced an approximately 310 bp amplicon for *C. krusei* strains (*n*=3). The *F. solani* strains (*n*=11) were amplified by *Fusarium* genus-specific primer pair ITS1- GSFus1 yielding approximately 450 bp amplicon. In the genus specific assay, the amplification product of the universal fungal primers, ITS1 and ITS4 was also detected, therefore the larger amplicon served to confirm the presence of a fungal target. A *Cryptococcus neoformans* isolate produced an amplicon of approximately 500 bp with the universal fungal primers, while the bacterial species tested were not detected. In the species differentiation assay, species specific primers together with the universal fungal primer ITS 1 were used to identify *A. terreus*, *C. glabrata* and *C. krusei*. A strain of *A. terreus* was amplified

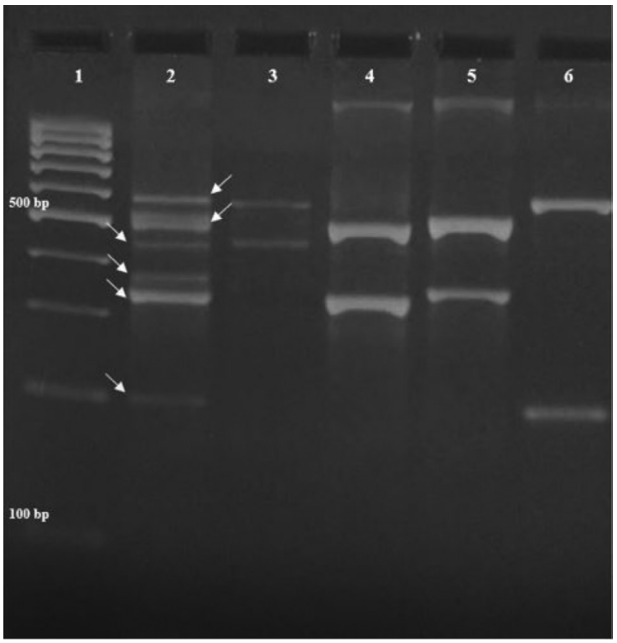

FIGURE 1. Genus-specific multiplex PCR with mixed genomic DNA of *F. solani*, *C. albicans*, *C. krusei* and *A. fumigatus* (lane 2) compared with single genomic DNA multiplex PCR as a reference (lanes: 3-6). Lane 2: Universal fungal detection (ITS1 & ITS4 primers) for (arrows indicate, respectively, from top to bottom) filamentous fungi (~550 bp) and yeast (~500 bp), genus specific detection of *F. solani* (~450 bp), *C. albicans* (~340 bp), *C. krusei* (~310 bp) and *A. fumigatus* (~200 bp). Lane 3: Universal fungal detection of *F. solani* (~550 bp) and genus specific amplicon (~450 bp), lane 4: *C. krusei* (~500 bp & ~310 bp), lane 5: *C. albicans* (~500 bp & ~340 bp) and lane 6: *A. fumigatus* (~550 bp & ~200 bp)

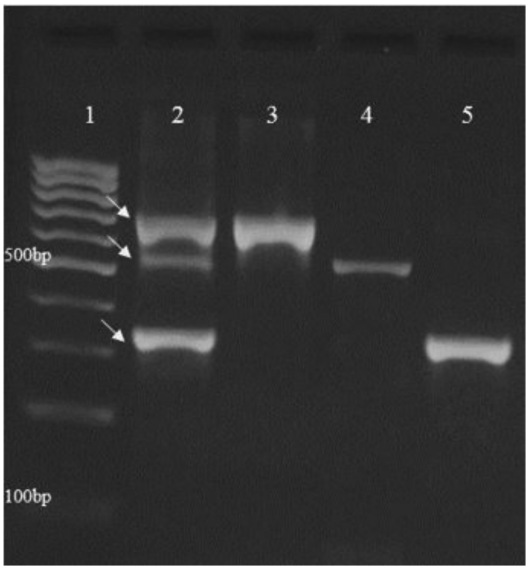

FIGURE 2. Species-specific multiplex PCR performed with mixed genomic DNA of *C. glabrata*, *A. terreus* and *C. krusei* (lane: 2) compared with single genomic DNA as a reference (lanes: 3-5). Lane 2: *C. glabrata* (~700 bp), *A. terreus* (~510 bp) and *C. krusei* (~310 bp). Lane 3: *C. glabrata* (~700 bp), lane 4: *A. terreus* (~510 bp), lane 5: *C. krusei* (~310 bp)

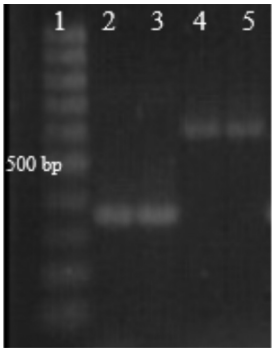

FIGURE 3. Genus-specific detection of *C. albicans* and C. *glabrata* performed in simplex PCR assay (primer ITS1-GSCand1) showing different sizes of amplicon. Lane 2-3: *C. albicans* (~340 bp) and lane 4-5: *C. glabrata* (~600 bp)

using the primer pair ITS1-SSAter1 yielding approximately 510 bp amplicon and 9 strains of *C. glabrata* were amplified by the *C. glabrata*-specific primer pair ITS1-SSCglab1 producing amplicons of approximately 700 bp. *C. krusei*-specific primer pair ITS1-SSCkrusei produced the same amplicon size as before, of 310 bp.

Optimum cycling conditions was achieved with annealing temperature set at 51.5ºC and extension temperature at 65ºC for 35 cycles. The components of the PCR reaction were also optimized with ITS 4 primer concentration reduced to 0.1 µM whereas other primers were retained at 0.4 µM. Under these optimum conditions the limit of detection (LOD) for the genus specific assay was 1 ng DNA for *A. fumigatus* and *C. albicans*, 0.1 ng DNA for F. *solani*, while the species *specific* assay detected 0.1 ng DNA of *A. terreus*, 10 pg DNA of *C. krusei* and *C. glabrata*.

DISCUSSION

In this study, a dual-panel multiplex PCR assay was developed and tested for its accuracy and sensitivity in detecting *Candida*, *Aspergillus* and *Fusarium* spp. isolates. Three primers for individual genus detection and another three primers for *C. glabrata*, *C. krusei* and *A. terreus* were designed and optimised for multiplex detection of the fungal targets.

The ITS 1- 5.8S- ITS 4 region was chosen for the design of genus and species-specific primers, due to high nucleotide variability among genera and species. The ITS region is a good molecular target for species level identification (Landlinger et al. 2009) and is extensively used as a universal DNA barcode in fungal taxonomy studies (Sulaiman et al. 2014). Furthermore, it is present at approximately 100 copies per genome (Henry et al. 2000) and is not found in

prokaryotic and human genomes (Than et al. 2012). The genus and species specific primers for genera and species levels identification were used in two separate assays to facilitate the interpretation of results and reduce reaction complexity. These two assays have the same PCR cycling conditions thus both reactions may be run together. The genus specific multiplex PCR reactions were optimized to simultaneously amplify two different loci by the genus specific primer with ITS 1 and ITS 1 with ITS 4, producing two distinctive amplicon sizes. The use of the universal fungal primers ITS1 and ITS 4 in this assay enables detection of any other fungal pathogen that may cause infections in patients, hence is an added advantage of this PCR assay.

The *Candida* genus-specific primer did not detect *C. krusei* strains which are genetically diversified from other pathogenic *Candida* spp. (Berrouane & Hollis 1996) and found to be 60 to 70% different in their DNA sequence pattern compared to other *Candida* spp. However, the non-amplification of *C. krusei* strains by *Candida*-genus specific primer and ITS 1 does not impact the outcome of this multiplex PCR assay as the *C. krusei*-specific primer, included in the reaction mix, detects *C. krusei* strains with amplicon size similar to *Candida* genus specific primer. *C. glabrata* strains are distinctive even in the genus-specific PCR producing the largest amplicon (approximately 600 bp) among targeted regions.

No cross reactivity was detected with other clinically important fungal, bacterial and human DNA by the BLAST program and subsequent experiments carried out with the *Aspergillus*, *Candida* and *Fusarium* genus-specific primer pairs and *Aspergillus terreus*, *C. glabrata* and *C. krusei* species-specific primer pairs. There was no amplification of non-target species isolates by these primers, as observed in gel electrophoresis, following extensive cross-amplification assays. This proves the high specificity of the primers to detect the respective genus and species making them suitable for use in a multiplex PCR assay. To confirm that the correct target was amplified, the PCR amplified product for each pair of primers used was sequenced and the results were as expected.

The species specific multiplex PCR assay targeted *C. krusei*, *C. glabrata* and *A. terreus* due to their reduced susceptibility or resistance to the major prescribed antifungal drugs, therefore their detection at the earliest stage will aid in appropriate selection of antifungal treatments for patients.

Current epidemiological trends recognize *A. terreus* as the second or the third most common etiological agent of invasive aspergillosis (Neal et al. 2011) especially in patients with haematological malignancies (Steinbach & Perfect 2003). *In vitro* and *in vivo* studies have demonstrated that isolates of *A. terreus* are associated with resistance to the antifungal drug amphothericin B, the empirical therapy for serious, invasive infections (Bašková & Buchta 2012). Furthermore, a recent study found a slight increase in invasive fungal infections due to non-*albicans Candida* infections, including *Candida glabrata* and *Candida krusei* in granulocytopenic and immunocompromised patients (Vazquez et al. 2013). *C. krusei* caused a higher mortality rate of 49% among immunocompromised patients as compared to 28% mortality by *C. albicans* infections (Richardson & Lass-Flörl 2008). *C. krusei* and *C. glabrata* were highly resistant to fluconazole (Chang et al. 2001) and less susceptible to itraconazole and amphotericin B (Abbas et al. 2000). The incidence of non-*albicans Candida* infections has been attributed to the widespread use of fluconazole as a prophylaxis antifungal (Cuenca-Estrella et al. 2008).

In order for PCR to be a sensitive diagnostic tool for invasive disease, a limit of detection (LOD) of <10 CFU/mL of blood should be achieved (Avni et al. 2011). The genus-specific multiplex PCR assay achieved an LOD of 0.1-1 ng DNA while the species-specific assay detected 10 pg-1 ng DNA. The fungal genome has an average size of approximately 0.038 pg or approximately 1.9 pg DNA in 50 cells (Gregory et al. 2007). Therefore, the sensitivity of the PCR assays should be increased, by increasing the number of PCR cycles to achieve the desired sensitivity for clinical application.

Conclusion

The multiplex PCR assays described here are able to differentiate three major clinically important fungal genera, namely *Candida*, *Aspergillus* and *Fusarium* and the resistant species *A. terreus*, *C. glabrata* and *C. krusei*. The deployment of a rapid, low cost yet reliable method for early diagnosis of invasive fungal infections is paramount to improving clinical management of the disease especially in hospitals with limited resources.

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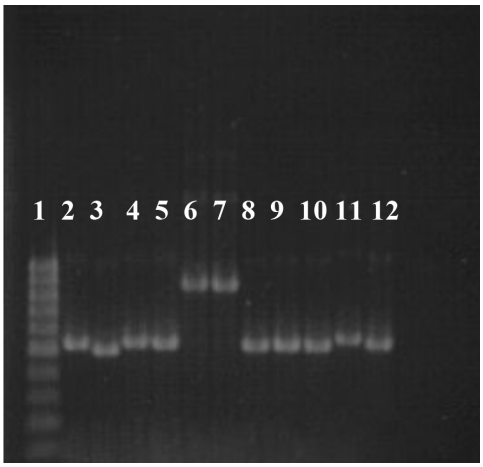
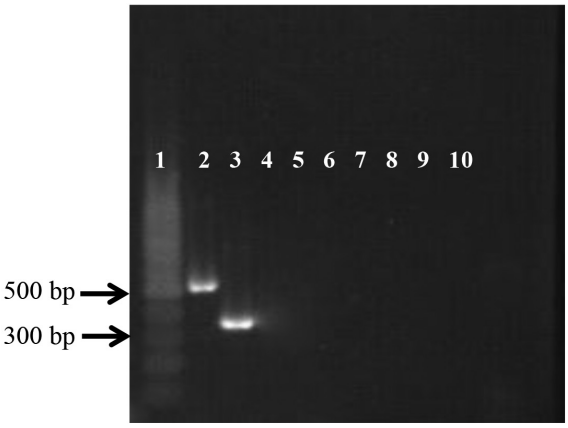
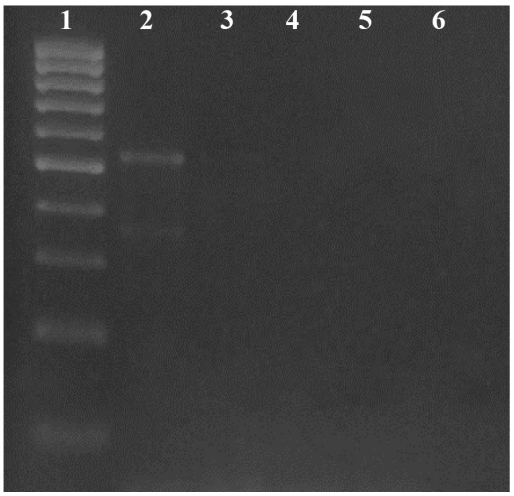
SUPPLEMENTARY FILE



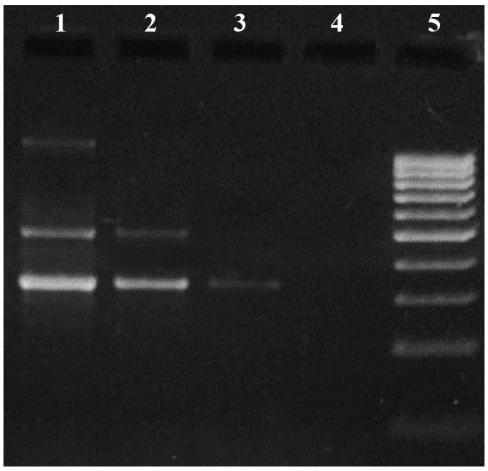
SUPPLEMENTARY 1. Gel electrophoresis of PCR product using DNA sample from *Candida* spp. isolates, with universal primers ITS 1 and ITS 4. Lane 1: 100 bp DNA ladder. Lane 2: *Candida albicans* MM 0692. Lane 3: *C. krusei* ATCC 6258. Lane 4: *C. dubliensis* MM 2524. Lane 5: *Candida albicans* MM 566. Lane 6: *C. glabrata* MM 0691. Lane 7: *C. glabrata* ATCC 66032. Lane 8: *C. parapsilosis* MM 0884. Lane 9: *C. parapsilosis* MM 6950. Lane 10: *C. parapsilosis* ATCC 20246. Lane 11: *Cryptococcus neoformans* D 13080. Lane 12: *C. albicans* ATCC 10231



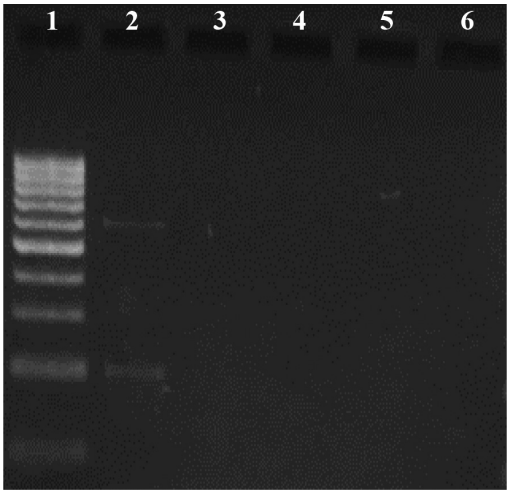
SUPPLEMENTARY 2. Gel electrophoresis of PCR product using set of universal primers ITS 1 & ITS 4 (Lane 2) and set of *Candida* genus-specific primer, GSCand1 & ITS 1 with DNA isolates of different species (Lane 3 to Lane 10). Lane 1: 100 bp DNA ladder. Lane 2: positive control, *C. albicans* MM 1076. Lane 3: positive control, *C. parapsilosis* MM 1113. Lane 4: *A. fumigatus* MM 956. Lane5: *A. niger* MM 2735. Lane 6: *A. flavus* MM 1938. Lane 7: *F. solani* MM 0020. Lane 8: *S. aureus*. Lane 9: *Syctalidium* sp. Lane 10: *E. coli*



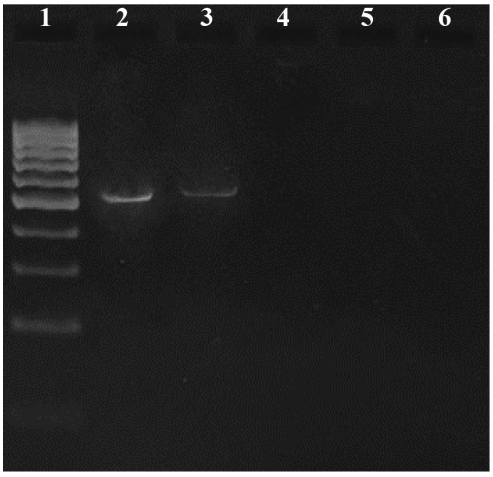
SUPPLEMENTARY 3. Gel electrophoresis of PCR product for sensitivity detection test using DNA isolate of *C. albicans* in multiplex PCR assay set 1. Lane 1: 100 bp DNA ladder. Lane 2: DNA concentration of 0.5 ng/µL. Lane 3: DNA concentration of 0.05 ng/µL. Lane 4: DNA concentration of 5.0 pg/µL. Lane 5: DNA concentration of 0.5 pg/µL. Lane 6: negative control



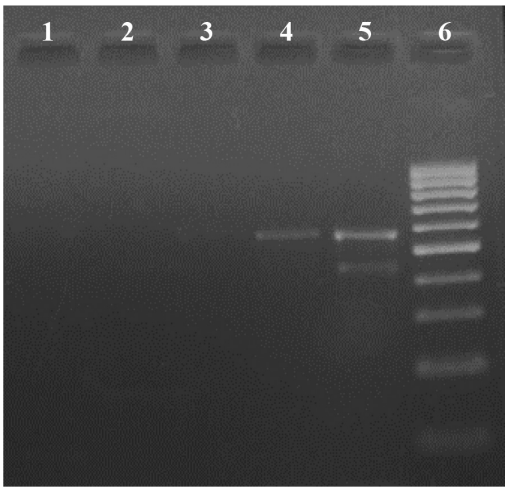
SUPPLEMENTARY 4. Gel electrophoresis of PCR product for sensitivity detection test using DNA isolate of *C. krusei* in multiplex PCR assay set 1. Lane 1: 100 bp DNA ladder. Lane 2: DNA concentration of 0.5 ng/µL. Lane 3: DNA concentration of 0.05 ng/µL. Lane 4: DNA concentration of 5.0 pg/µL. Lane 5: DNA concentration of 0.5 pg/µL. Lane 6: negative control



SUPPLEMENTARY 5. Gel electrophoresis of PCR product for sensitivity detection test using DNA isolate of *A. fumigatus* in multiplex PCR assay set 1. Lane 1: 100 bp DNA ladder. Lane 2: DNA concentration of 0.5 ng/µL. Lane 3: DNA concentration of 0.05 ng/µL. Lane 4: DNA concentration of 5.0 pg/µL. Lane 5: DNA concentration of 0.5 pg/µL. Lane 6: negative control



SUPPLEMENTARY 6. Gel electrophoresis of PCR product for sensitivity detection test using DNA isolate of *A. terreus* in multiplex PCR assay set 2. Lane 1: 100 bp DNA ladder. Lane 2: DNA concentration of 0.5 ng/µL. Lane 3: DNA concentration of 0.05 ng/µL. Lane 4: DNA concentration of 5.0 pg/µL. Lane 5: DNA concentration of 0.5 pg/µL. Lane 6: negative control



SUPPLEMENTARY 7. Gel electrophoresis of PCR product for sensitivity detection test using DNA isolate of *F. solani* in multiplex PCR assay set 1. Lane 1: 100 bp DNA ladder. Lane 2: DNA concentration of 0.5 ng/µL. Lane 3: DNA concentration of 0.05 ng/µL. Lane 4: DNA concentration of 5.0 pg/µL. Lane 5: DNA concentration of 0.5 pg/µL. Lane 6: negative control