Malays J Pathol 2023; 45(3) : 397 – 403

ORIGINAL ARTICLE

Species-specific PCR primers for simultaneous detection of *Aspergillus fumigatus*, *Aspergillus terreus*, *Candida albicans* and *Candida glabrata* in invasive fungal infections

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Abstract

A rapid and accurate diagnosis of invasive fungal infections (IFIs) has been a great challenge particularly in cases requiring prompt antifungal treatment. In this study, four primer pairs were designed for a quadruplex PCR assay, which was developed for detection of four fungal species simultaneously. DNA extraction of cultured colonies and spiked blood samples were performed using conventional (phenol-chloroform) techniques and commercial DNA extraction kit. The optimum annealing temperature for this assay was 60°C. The assay was able to amplify all four genes and showed 100% specificity. No amplification of any genes was obtained against other species (n=14), which included two bacteria species. In conclusion, this quadruplex PCR assay is specific, rapid and reliable to detect *A. fumigatus*, *A. terreus*, *C. albicans* and *C. glabrata* simultaneously.

Keywords: invasive fungal infection, quadruplex PCR, fungal diagnosis

INTRODUCTION

Recently, invasive fungal infections (IFIs) has shown enormous increase in prevalence and become a major cause of infectious morbidity and mortality among immunocompromised, organ transplant and HIV patients.[1] It is estimated that around 1.5 million IFIs were reported annually worldwide where more than 80% of which was attributed to infections involving *Candida*, *Aspergillus*, or *Cryptococcus* spp.[2]

In many developed countries, *C. albicans* is the most prevalent pathogenic agent in clinical settings where cases of candidemia increased almost 5-fold in the last 10 years in general.[3] However, non-*albicans Candida* such as *C. glabrata*, *C. tropicalis*, *C. parapsilosis* and *C. krusei* had also been reported in more than 50% isolates in most clinical setting.[4] Furthermore, *C. glabrata* has been reported as the second most common in candidemia which has increased almost up to 25-30% and mostly found in patients aged more than 60 years.[4,5] In Malaysia setting, Khaithir *et al*. reported that *Candida* species were the most frequently isolated fungal organisms associated with IFIs, closely followed by *Aspergillus* species.[6] *Aspergillus* spp. such as *A. fumigatus*, *A. flavus*, *A. terreus* and *A. niger* were discovered among other common pathogens in IFIs.[7]

Despite the significant impact of IFIs on patients' morbidity and mortality, effective diagnosis of this condition is often difficult. Fungal culture and microscopy, which is the gold standard for IFIs diagnosis is time consuming and has low sensitivity.[1,4] As rapid and accurate detection is a key for appropriate treatment, it is mandatory for laboratory service to provide alternative methods for detection of IFIs.

Molecular methods have been progressively developed to improve the detection of pathogenic fungi and overcome the limitations of conventional methods. PCR-based methods have been shown to be promising due to their robust simplicity, sensitivity and specificity in diagnostic settings. However, there is no established, standardised methods available for the simultaneous detection of *Candida* spp. and *Aspergillus* spp. in Malaysia.

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In this study, we designed a species-specific primers for PCR assay to detect the common pathogenic fungi simultaneously using multiplex PCR method. The assay combined four species-specific genes using the multiplex PCR to detect *A. fumigatus*, *A. terreus*, *C. albicans* and *C. glabrata*, together with an internal amplification control.

MATERIALS AND METHOD

Primer design and target selection

Species-specific primer pairs were designed using IDT DNA PrimerQuest online tool (https://sg.idtdna.com/PrimerQuest/) based on the housekeeping gene (GenBank accession no.: *bgt1* gene (AF038596), *benA* gene (KF669507), ITS2 gene (AJ853768), *LEU2* gene (CP048125). The *in-silico* specificity of these primers was assessed using Primer-BLAST for selected DNA regions. Homo-dimer, hetero-dimer and hairpin formation of primers were measured for the simultaneous detection, as the heterodimer of delta G values were ≤ -9 kcal/mol of all primers. These primers were then synthesised by IDT, Singapore and their amplicon sizes were shown in Table 1.

Fungal and bacterial isolates

The filamentous mould and yeast strains were obtained from Mycology Laboratory of Medical Microbiology & Parasitology Department, Hospital Universiti Sains Malaysia (HUSM) and American Type Culture Collection (ATCC) strains, which included *A. fumigatus* ATCC 46645, *A. terreus*, *A. flavus*, *C. albicans* ATCC 64124, *C. glabrata*, *C. tropicalis*, *C. orthopsilosis*, *Cryptococcus neoformans*, *Paecilomyces variotii* and *Trichophyton rubrum*. Two bacterial strains, *Klebsiella pneumoniae* (ATCC 1706) and *Staphylococcus aureus* (ATCC 25923) were used in specificity evaluation of the developed multiplex PCR. Species identification of the fungal strains was validated by conventional morphological examination and ribosomal RNA gene sequencing beforehand.

Fungal suspensions for spiked blood preparation

The fungal isolates were subcultured on Sabouraud Dextrose Agar (SDA) (Oxoid, UK) plates and incubated at 30°C. As for *C. albicans* and *C. glabrata*, the fungal suspension were prepared from fresh, mature (2- to 3-day-old) cultures. As for *A. fumigatus* and *A. terreus*, the colonies were covered with 5-7 ml of sterile distilled water containing 1% Tween 20. The

TABLE 1: List of primer pairs designed to amplify pathogenic fungal DNA

Species	Amplicon size (bp)	Primer sequences (5'-3')	GC content (%)	T_m (°C)	References
A. fumigatus	149	*bgt1*_F- GCT GCT GCC TCC AAG AAT G	57.9	56.8	This study
		*bgt1*_R- GCA GTC ACT CGC GGA GTA G	63.2	57.8	
A. terreus	147	*benA*_F- GGC TCC CAT AAT GGA GGT TTA C	50.0	55.3	This study
		*benA*_R- GTG AAG AAT CTG TCC CAG GAT G	50.0	55.2	
C. albicans	103	ITS2_F- GTT TGC TTG AAA GAC GGT AGT G	45.5	54.3	This study
		ITS2_R- AAG ATA TAC GTG TGG ACG TTA C	40.9	52.0	
C. glabrata	157	*LEU2*_F-GTT AGA GAA CTA GTG GGT GGT ATT T	40.0	54.1	This study
		*LEU2*_R-TAG GTA AAG GTG GGT TGT GTT G	45.5	54.8	

conidia were scrapped using sterile scalpel and filtered to a sterile tube with sterile gauze using filters with a pore diameter 11 μm to remove the hyphae, mycelium or clumps. Then, appropriate dilutions were performed in order to achieve the optimal concentration for haemocytometer cell-counting (Neubauer chamber, Weber Scientific, UK). The final inoculum concentration was adjusted to a range of 1.0-5.0 x 10^7 spores/ml by haemocytometer counting. All adjusted suspensions were verified by plate count on SDA of 25, 50 and 100 μl suspension volumes. The target inoculum size range was established around ~1.0-5.0 x 10^7 cfu/ml. The suspension was then spiked into uninfected EDTA whole blood samples which intended to mimic invasive or blood stream infection.

Genomic DNA extraction from fungal colonies

The fungal isolates were grown on SDA at 30°C for 2 to 5 days. Conidia and hyphae were harvested and stored at -80°C until further use. Fungal DNA was extracted using the phenol-chloroform method. Briefly, 500 μl lysis buffer (1.0% SDS, NaCl, 0.5 M EDTA, 1 M Tris-HCl [pH 8]) with 5 μl 2-mercaptoethanol (Sigma-Aldrich, UK) were added into frozen fungal mycelia and yeast colonies and macerated using pellet pestle. The mixture was vortexed vigorously and incubated at 65°C for 90 min. After incubation, phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma Aldrich, UK) was added to the mixture and mixed by inverting the tubes for 2 min. The mixture was centrifuged at 20 000 xg for 5 min. The uppermost aqueous layer was transferred to a new 1.5 ml tube and mixed with an equal volume of isopropanol and left for precipitate for at least 2 h. Then, the new mixture was centrifuged at 20 000 xg for 5 min, resulting in DNA pellet formations at the bottom of the tube. Next, a washing step was repeated twice with 70% (v/v) ethanol. Finally, DNA pellets were dried at room temperature by dissolving in sterile nuclease-free water and stored at -20°C.

Whole blood DNA extraction

For multiplex PCR method, a spiked blood specimen was used where 300 μl of whole blood was added in 200 μl of AL buffer of DNeasy Blood & Tissue kit (QIAGEN Inc., Germany). The following steps were continued according to the manufacturer's instructions. For the elution step, 50 μl of AE buffer was added to the center of the column.

PCR amplification

Monoplex and quadruplex PCRs were performed using MyTaq™ Red DNA Polymerase mastermix reagent (Bioline, UK). Both assays were amplified with 20 ng of genomic DNA in a 25 μl reaction volume containing 5 μl of 5X MyTaq Red Buffer (5 mM deoxynucleotide triphosphates (dNTPs), 15 mM $MgCl_2$, stabilizers, enhancers), 0.5 μl of each primer, 0.2 μl MyTaq™ Red DNA Polymerase and 2 μl of fungal DNA. The PCR parameter consisted of pre-incubation step for 5 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 60°C and 30 s at 72°C with a final extension step of 5 min at 72°C. The annealing temperature (T_a) has undergone prior optimisation using temperature range of 55-65°C. The PCR products were analysed using 2.0% agarose gel in 0.5x TBE electrophoresis buffer (First BASE Ptd. Ltd, Singapore). The gel electrophoresis was run at 80V for 90 min to separate the bands.

Analytical specificity of the monoplex and quadruplex PCR assays

To evaluate the efficiency of the monoplex and quadruplex PCR assay, extracted DNA from fungal isolates: *A. fumigatus* ATCC 46645, *A. terreus*, *A. flavus*, *A. nidulans, C. albicans* ATCC 64124, *C. glabrata*, *C. tropicalis*, *C. parapsilosis, C. orthopsilosis*, *C. neoformans*, *P. variotii* and *T. rubrum* and two bacterial isolates: *K. pneumoniae* and *S. aureus* were subjected to both monoplex and quadruplex PCR assays.

RESULTS

Performance of monoplex assays

The primers pairs were tested in monoplex PCR using different strains to confirm their specificity. An initial validation of the PCR test's performance used (n=10) to validate the designed primers. All the designed primers for detecting *A. fumigatus*, *A. terreus*, *C. albicans* and *C. glabrata* were successfully amplified *bgt1* gene (149 bp), *benA* gene (147 bp), ITS2 gene (103 bp) and *LEU2* gene (157 bp). All of the four genes amplified using this quadruplex PCR assay were shown in Figure 1. The bands were distinguished according to their expected sizes on the gel electrophoresis.

Performance of quadruplex assay

The developed quadruplex PCR assay successfully amplified *bgt1* gene (149 bp), *benA* gene (147 bp), ITS2 gene (103 bp), *LEU2* gene (157 bp) and *glmM* gene (105 bp) of IAC. The

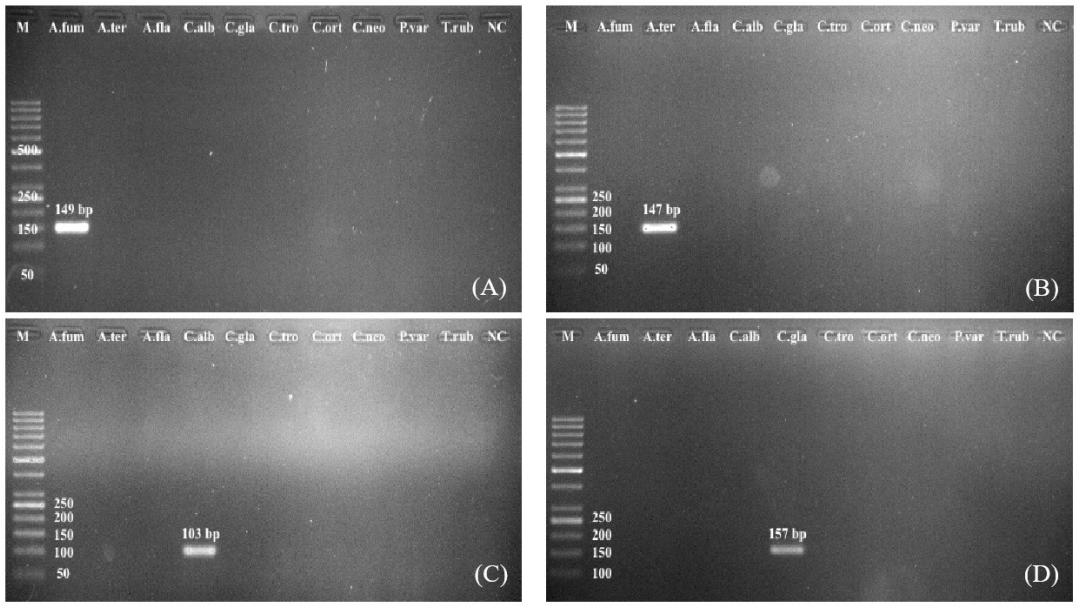

FIG. 1: Gel electrophoresis of monoplex PCR amplicons generated from *bgt1* gene (A), *benA* gene (B), ITS2 gene (C) and *LEU2* gene (D). A.fum – *Aspergillus fumigatus*, A.ter – *Aspergillus terreus*, A.fla – *Aspergillus flavus*, C.alb – *Candida albicans*, C.gla – *Candida glabrata*, C.tro - *Candida tropicalis*, C.ort – *Candida orthopsilosis*, C.neo – *Cryptococcus neoformans*, P.var – *Paecilomyces variotii*, T.rub – *Trichophyton rubrum*, NC – negative control, M – 50 bp DNA ladder.

optimum T_a used in this assay was 60°C (detail optimisation data not shown). Multiplex PCR of four target genes [*bgt1* gene (149 bp), *benA* gene (147 bp), ITS2 gene (103 bp), *LEU2* gene (157 bp)] showed that the primers specifically amplified *A. fumigatus*, *A. terreus*, *C. albicans* and *C. glabrata* simultaneously as presented in Figure 2(a) and 2(b). The internal amplification control (IAC) of 105 bp amplicon size was demonstrated in all reactions. No amplification seen in *A. flavus*, *A. nidulans*, *C. tropicalis*, *C. parapsilosis*, *C. orthopsilosis*, *C. neoformans*, *P. variotii*, *T. rubrum*, *K. pneumoniae* and *S. aureus*, denoting the specificity of the assay.

DISCUSSION

Specific detection of fungal pathogen in clinical specimens has been the main problem in the management of invasive fungal infections, which relies on the accurate and timeliness of the diagnostic tools.[9,10] In this study, we demonstrated the use of specific primers in a multiplex PCR assay which is capable of detecting four common fungal species *A. fumigatus*, *A. terreus*, *C. albicans* and *C. glabrata*. These primers were designed based on *bgt1* gene, *benA* gene, ITS2 gene and *LEU2* gene.

The rRNA gene cluster has been used as targets in previous reports, namely D1/D2 domain of LSU rDNA, 28S rRNA, 18S rRNA and ITS regions.[1,11] Both ITS and 28S genes were substantially used instead of other DNA markers for fungal detection and identification.[12,13] However, overlapping of the molecular targets, which interferes with the correct identification has been reported.[14] In addition, other protein-coding markers namely beta-tubulin, translation elongation factor 1-∝ gene, or actin and housekeeping genes can also be used in other fungal identification.[15,16] To our knowledge, this present study is the first study on the development of multiplex PCR using these housekeeping genes (*bgt1*, *benA*, ITS2, *LEU2*) which simultaneously detects four common fungal pathogens in clinical setting. This assay has a great potential as rapid diagnostic tool with short turn-around-time (<4 hours per run).

Primer designing is a critical process in development of specific assay. The paucity of fungal genome database further enhanced the challenge in identifying conserve regions and curate specific primers. For detection of *A. fumigatus*, ß-1,3-glucanosyl transferase (*bgt1*) gene was selected. Glucanosyltransferases modify the structure of the cell wall and the enzyme's activity is encoded by *bgt1* gene which has been biochemically identified in the *A. fumigatus* cell wall autolysate.[17] For *A. terreus*,

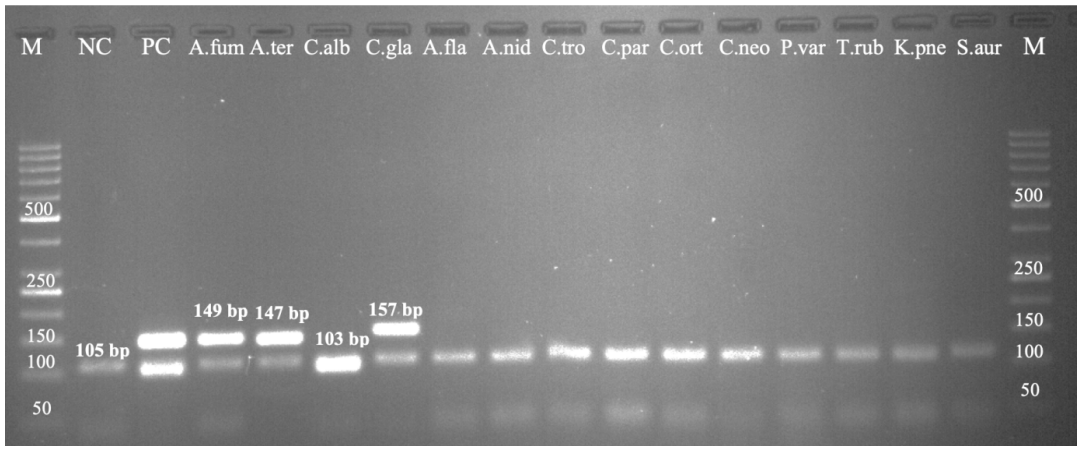

FIG. 2 (a): Gel electrophoresis of multiplex PCR amplicons of *bgt1* gene, *benA* gene, ITS2 gene and *LEU2* gene from culture samples. A.fum – *Aspergillus fumigatus*, A.ter – *Aspergillus terreus*, C.alb – *Candida albicans*, C.gla – *Candida glabrata*, A.fla – *Aspergillus flavus*, A.nid – *Aspergillus nidulans*, C.tro – *Candida tropicalis*, C.par – *Candida parapsilosis*, C.ort – *Candida orthopsilosis*, C.neo – *Cryptococcus neoformans*, P.var – *Paecilomyces variotii*, T.rub – *Trichophyton rubrum*, K.pne – *Klebsiella pneumoniae*, S.aur – *Staphylococcus aureus*, NC – negative control (include IAC), PC – positive control, M – 50 bp DNA ladder.

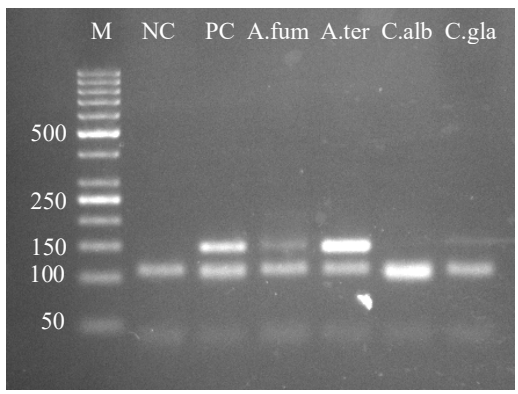

FIG. 2 (b): Gel electrophoresis of multiplex PCR amplicons of *bgt1* gene, *benA* gene, ITS2 gene and *LEU2* gene from spiked blood samples. A.fum – *Aspergillus fumigatus*, A.ter – *Aspergillus terreus*, C.alb – *Candida albicans*, C.gla – *Candida glabrata*, NC – negative control (include IAC), PC – positive control, M – 50 bp DNA ladder.

beta-tubulin (*benA*) gene was selected, which encodes for polypeptide proteins in eukaryotes and has been used for fungal phylogenetic analysis.[18,19] In addition, *benA* gene acts on various metabolic pathways and gene express regulation in fungi.[18] The ITS2 of rRNA gene was chosen to detect *C. albicans* because of its functions in cell viability and its validated qPCR application in previous study.[20] For *C. glabrata*, isopropylmalate dehydrogenase (*LEU2*) gene was selected as target due to its inclusion in *C. glabrata* multilocus sequence typing (MLST) scheme, and was shown to be specific for *C. glabrata*.

Another main concern in developing a PCR-based assay is the optimisation of PCR protocols. The precision of a multiplex assay depends on diligent optimisation of every conditions of the PCR. Using the designed primer pairs and PCR protocols as mentioned in 'Results' section, the performance of this assay was promising with 100% specificity. These targets and primer pairs will be applied in assays of different test formats. In this study, the limit of detection (LOD) was not fully performed as the developed assay will be converted to real-time PCR assay, in which the LOD is expected to be much better than conventional PCR. Conversion to real-time PCR

also explains the small amplicon sizes (less than 200bp) produced in this assay.

Effective fungal DNA extraction is also one of the major factors in determining sensitivity of a molecular assay. In this study, fungal DNA was extracted from two different types of specimens; cultured colonies and spiked blood specimens. Besides, the extraction method for filamentous fungi especially, is complex where as having high extracted DNA quality and purity is an achievement. This study optimised the extraction method to extract the filamentous fungal specifically. Both conventional (phenol-chloroform method) and commercial kits (QIAGEN) were used for culture and spiked blood samples and they gave good results despite any inhibitions (e.g., heparin) were expected at first which may disrupt the PCR kinetics.[21] Besides, *glmM* gene of *H. pylori* was used in our assay as an exogenous internal amplification control (IAC) to confirm DNA amplification, detect any false negative and detect the presence of amplification inhibitory substances in any samples in the multiplex PCR assay.[22]

As demonstrated in Table 1 and Figure 2(a), the amplicon size of the four genes are almost the same (147 bp, 149 bp and 157 bp), which could not be differentiated on agarose gel. This was purposely designed as this quadruplex PCR assay will be converted to real-time PCR assay. Given that the primers have successfully amplified all four genes in monoplex and quadruplex assays by conventional format, they should equally be effective (if not better) in real-time PCR, which is a more robust technique with much improved sensitivity.

In conclusion, the designed primers and quadruplex PCR assay developed in this study have shown successful specific amplification of *A. fumigatus*, *A. terreus*, *C. albicans* and *C. glabrata*, from both cultured colonies and spiked blood samples. These findings significantly contribute to the betterment of laboratory diagnosis and management of invasive fungal infections.

Acknowledgement: We would like to acknowledge the Mycology Laboratory, Department of Medical Microbiology and Parasitology for the access to laboratory. This study was supported by Universiti Sains Malaysia RUI grant 1001/PPSP/812206 awarded to Azian Harun.

Authors contribution: Conception and design: Azian Harun. Conduct of experiments, data analysis and interpretation: Yasmin Khairani Muhammad Ismadi. Drafting the manuscript and/or revising the manuscript: Yasmin Khairani Muhammad Ismadi, Suharni Mohamad, Azian Harun.

Conflict of interests: The authors declare no conflict of interests.

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