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Full Length Research Paper

Simplex and triplex polymerase chain reaction (PCR) for identification of three medically important *Candida* species

Nabil S. Harmal[1,2], Alireza Khodavandi[3], Mohammed A. Alshawsh[4], Farida Jamal[1], Zamberi Sekawi[1], Ng Kee Peng[5] and Pei Pei Chong[6]*

[1]Department of Medical Microbiology and Parasitology, Faculty of Medicine and Health Sciences, University Putra Malaysia, 43400 Selangor, Malaysia.
[2]Department of Medical Microbiology, Faculty of Medicine and Health Sciences, Sana'a University, Sana'a, Yemen.
[3]Department of Paramedical Sciences, Gachsaran Branch, Islamic Azad University, Gachsaran, Iran.
[4]Department of Molecular Medicine, Faculty of Medicine, University of Malaya, 50603, Kuala Lumpur, Malaysia.
[5]Department of Medical Microbiology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia.
[6] Department of Biomedical sciences, University Putra Malaysia, Selangor, Malaysia, 43400, Malaysia.

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***Candida* species are a major cause of invasive infections in both critically ill and immunocompromised patients. Hence, rapid identification of these pathogens may facilitate specific therapy and patient management. The development of rapid and specific diagnostic methods remains a challenge. Herein, we developed the simplex and triplex polymerase chain reaction (PCR) for the identification of three medically important *Candida* species namely *C. albicans*, *C. parapsilosis* and *C. tropicalis*. The developed methods target the phospholipase B gene (*PLB*). The primers designed achieved highly specific identification of the selected species using both the simplex PCR and the triplex PCR formats, which were confirmed by DNA sequencing. The primers did not show any non-specific amplification when tested with DNA from other *Candida* species and other fungal species such as *Aspergillus* and *Cryptococcus*. These results showed that the *PLB* gene provides a novel target that could be used for the detection of medically important *Candida* species from clinical specimens.**

Key words: *Candida* species, primers, phospholipase B gene (*PLB*), polymerase chain reaction (PCR).

INTRODUCTION

The genus *Candida* is a major cause of invasive infections in both immunocompromised and critically patients. It is considered the fourth most common cause of hospital acquired blood-borne infection. *Candida albicans* remains the most commonly isolated species, and together with *Candida tropicalis* and *Candida parapsilosis* comprises the most medically important *Candida* species (Enoch et al., 2006). Therefore, the rapid identification of the pathogenic *Candida* species will facilitate optimal antifungal treatment and patient management. Conventional diagnostic approaches to the identification of fungal species are mainly based on phenotype analysis of fungal cultures. Nevertheless, these approaches are time-consuming and fail to discriminate between the pathogenic fungal species; information that is crucial for initiating specific antifungal therapy (Rickerts et al., 2007). A variety of molecular methods have been developed for the identification of medically important *Candida* species. Many of these

*Corresponding author. E-mail: cpp_labs@yahoo.com. Tel: +60-89472302. Fax: +60-89436178.

Abbreviations: ITS, Internal transcribed spacer; **PCR,** polymerase chain reaction; **ATCC,** American type culture collection; **SDA,** Sabouraud dextrose agar.

Table 1. List of the ATCC fungal strains used in this study.

Species	Strain*
Candida albicans	ATCC 14053
Candida dubliniensis	ATCC MYA-178
Candida famata	ATCC 62894
Candida glabrata	ATCC 2001
Candida guilliermondii	ATCC 6260
Candida kefyr	ATCC 66028
Candida krusei	ATCC 6258
Candida lusitaniae	ATCC 66035
Candida metapsilosis	ATCC 96144
Candida parapsilosis	ATCC 22019
Candida rugosa	ATCC 10571
Candida orthopsilosis	ATCC 96139
Candida tropicalis	ATCC 750
Aspergillus fumigatus	ATCC 36607
Aspergillus niger	ATCC 16888
Aspergillus terreus	ATCC 1012
Aspergillus flavus	ATCC 10124
Cryptococcus neoformans	Clinical Strain 001
Cryptococcus humicolus	Clinical Strain 002

*ATCC, American Type Culture Collection, Rockville, MD, USA.

assays are real-time quantitative polymerase chain reaction (PCR) tests, targeting several genes with the ribosomal multicopy gene (rDNA gene) being the most popular targeted genes (Schabereiter-Gurtner et al., 2007; Wellinghausen et al., 2009). A number of detection methods are used for identification of the PCR products currently used in the clinical microbiology laboratories (Boyanton et al., 2008). However, most methods are either expensive or laborious and clinically not applicable (Putignani et al., 2008). Therefore, the establishment of simple, inexpensive molecular approaches that are readily adaptable to the clinical microbiology laboratory remains a challenge.

Invasion of host cells by microbes entails penetration and damage of the outer cell envelope. Evidence implicating phospholipases in host cell penetration, injury, and lysis by microorganisms has been reported for *C. albicans*. The gene encoding *C. albicans* phospholipase B1 (PLB1) has been cloned (Hoover et al., 1998). In a previous study, we cloned and sequenced the *PLB* gene for several *Candida* species (Cheang et al., 2006). In contrast to the internal transcribed spacer (ITS) regions, the *PLB* gene of most *Candida* species showed surprisingly large sequence variation. Hence, we postulated that it would very likely be able to discriminate between the different species, in addition to being novel. The primers designed from the *PLB* gene will definitely be novel. Here, we developed assays specific for the fungal *PLB* gene, including the development of triplex PCR method, allowing the simultaneous identification and differentiation of three most medically important *Candida* species, namely *C. albicans, C. parapsilosis,* and *C. tropicalis*.

MATERIALS AND METHODS

Source of fungal species

American type culture collection (ATCC) reference strains of *Candida* species (n = 13), *Aspergillus* species (n = 4) and two clinical isolates of *Cryptococcus* species obtained from University Malaya Medical Centre (UMMC) were used in this study to confirm the specificity of the designed primers (Table 1).

Culture of fungal species

The fungal strains were streaked in the Sabouraud dextrose agar (SDA) plate and incubated at 37°C for 24 h; 48 h and 5 days for *Candida* species, *Cryptococcus* species and *Aspergillus* species, respectively. A single colony of the fungal organism was transferred from SDA plate and cultured in SDB for 24 and 48 h before proceeding to the DNA extraction.

DNA extraction and PCR amplification

The DNA extraction for the fungal species from culture broth was carried out using Wizard ® DNA Purification Kit (Promega, USA). Subsequently, the amplification of all extracted DNA samples was performed using the universal fungal primers ITS1 (5'- TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC-3') to ensure that the appropriate fungal target DNA was obtained. The ITS1 and ITS4 primer pair flanks a region of common sequences among all fungal species (White et al., 2009). The amplification condition contained 30 cycles, and PCR products were performed by gel electrophoresis, and then visualized through the AlphaImager HP imaging system.

Designing the specific primer from *PLB* gene

C. albicans, C. parapsilosis and C. tropicalis were selected as the organisms of interest and the nucleotide sequences of their *PLB* genes were downloaded from the GenBank database (http:// www.ncbi.nlm.nih.gov/). Table 2 shows the accession numbers and sources of the sequences used in this study. The *PLB* gene sequences from different *Candida* species were aligned and compared to find out the unique region of each species, assisted by ClustalW sequence alignment software. The species-specific primers for simplex PCR and multiplex PCR were designed based on the most unique sequences that can be found between the *Candida* species from the *PLB* gene. For multiplex PCR, the primers were selected to have relatively close annealing temperature to be reliable for the multiplex. The sequences of the designed primers were compared with the GenBank database using blastn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to ensure their specificities for the corresponding *Candida* species before being chemically synthesized. All primers were synthesized by AIT-Biotech (Singapore).

Simplex PCR amplification using species-specific primers

In a sterile PCR tube, 0.5 μg of extracted DNA was added to the

Table 2. List of *Candida* species and the accession number of their *PLB* gene sequences that were used in this study.

***Candida* strain**	**Sequence length**	**Sequence**	**Accession number**	**Source**
Candida albicans	1818 bp	Complete	XM_708729	Jones et al. (2004)
Candida glabrata	1980 bp	Complete	AF498581	Unpublished
Candida parapsilosis ATCC	527 bp	Partial 5'end	AY544773	Previous study*
Candida parapsilosis ATCC	546 bp	Partial 3'end	AY544774	Previous study*
Candida parapsilosis clinical strain	546 bp	Partial 3'end	AY544775	Previous study*
Candida krusei ATCC	527 bp	Partial 5'end	AY544762	Previous study*
Candida tropicalis ATCC	595 bp	Partial 5'end	AY394565	Previous study*
Candida tropicalis ATCC	423 bp	Partial 3'end	AY389800	Previous study*
Candida tropicalis clinical strain	594 bp	Partial 5'end	AY394566	Previous study*
Candida tropicalis clinical strain	423 bp	Partial 3'end	AY389801	Previous study*

*Previous study (Cheang et al., 2006).

mixture of 1× PCR buffer, 1.5 mM of $MgCl_2$, 0.08 mM of dNTPs (Promega, USA), 0.4 pmol/μl of species-specific forward and reverse primers (AIT-Biotech, Singapore), 0.02 U of Go Taq DNA polymerase (Promega, USA) and sterile ultrapure water to make up a total volume of 25 μl. The reaction was started with the initial denaturation at 95°C for 5 min and followed by 30 cycles of denaturation, annealing and extension at 95°C for 30 s, X°C (X°C = the optimal annealing temperature for each primer set) for 35 s and 72 °C for 1 min, respectively. The reaction was terminated after final extension at 72°C for 10 min. Hot-start PCR was used, whereby the DNA polymerase was added to the reaction mixture after 5 min of initial denaturation. The PCR products were visualized under UV light after gel electrophoresis and compared with the expected band size of the corresponding *Candida* species. The PCR products were then purified using QIAquick PCR Purification Kit (Qiagen, Germany). Eventually, the purified PCR products were sent to a commercial company (First BASE, Malaysia) and subjected to automated sequencing on both strands, using the specific forward and reverse primers for corresponding species of the simplex PCR. For the specificity test of the simplex PCR, a number of PCR experiments were run using a DNA template from the extracted DNA of the ATCC reference strains of *Candida* species (n = 13), *Aspergillus* species (n = 4), and also two clinical isolates of *Cryptococcus* species, to verify the ability of the designed primers to amplify only the DNA segment of the corresponding *Candida* species specifically.

Triplex PCR amplification using species-specific primers

In a sterile PCR tube, 0.5 μg of extracted DNA was added to the mixture of 1× PCR buffer, 2.0 mM of $MgCl_2$, 0.16 mM of dNTPs (Promega, USA) and 20 pmol/μl of a mixture of three pairs of the specific forward and reverse primers (AIT-Biotech, Singapore), respectively. Subsequently, 0.04 U of Go Taq DNA polymerase (Promega, USA) and sterile ultrapure water was added to get the total volume of 25 μl. The reaction was started with the initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation, annealing and extension at 95°C for 40 s, 50°C for 35 s and 72°C for 1 min, respectively. The reaction was terminated after final extension at 72°C for 10 min. Hot-start PCR was used whereby the DNA polymerase was added to the reaction mixture after 5 min of initial denaturation. The PCR products were used to run the agarose gel electrophoresis to test the specificity of each primer pair to the corresponding *Candida* species and visualized by AlphaImager HP imaging system. Eventually, the PCR products were sequenced to confirm the specificity of the primers (Life Technologies Corporation). For the specificity test of the multiplex PCR, a number of PCRs run, using a DNA template from the extracted DNA of the ATCC reference strains of *Candida* species (n = 13), *Aspergillus* species (n = 4) and the two clinical isolates of *Cryptococcus* species, were used as explained earlier in the simplex PCR.

RESULTS

PCR amplification using ITS1 and ITS4 primers

Due to the variability in length between both the ITS 1 and ITS 2 regions of different *Candida* species, amplicons which are different in size can be produced during the PCR. As shown in Figure 1A, the PCR products of *C. albicans, Candida glabrata, C. parapsilosis* and *C. tropicalis* ATCC strains, also *C. albicans* clinical isolate generated using ITS 1and ITS 4 universal fungal primers, were approximately 530, 870, 515, 520, and 530 bp, respectively. The ITS 1 and ITS 4 PCR amplification results of other *Candida* species ATCC strains such as *Candida famata, Candida guilliermondii, Candida lusitaniae, Candida metapsilosis* and *Candida orthopsilosis* are shown in Figure 1B, with product sizes of approximately 625, 610, 370, 517 and 513 bp, respectively. In addition, the ITS 1 and ITS 4 PCR products of *Cryptococcus neoformans* 001 (clinical isolate), *Cryptococcus humicolus* 002 (clinical isolate), *Aspergillus fumigatus, Aspergillus flavus*, *Aspergillus niger* and *Aspergillus terreus* ATCC strains are also shown in Figure 1C, and the product sizes were approximately 555, 550 bp for *Cryptococcus* species and 600 to 610 bp for *Aspergillus* species, respectively.

Simplex, triplex PCR and primers design

A simplex and a triplex PCR method were both developed to selectively identify and differentiate between

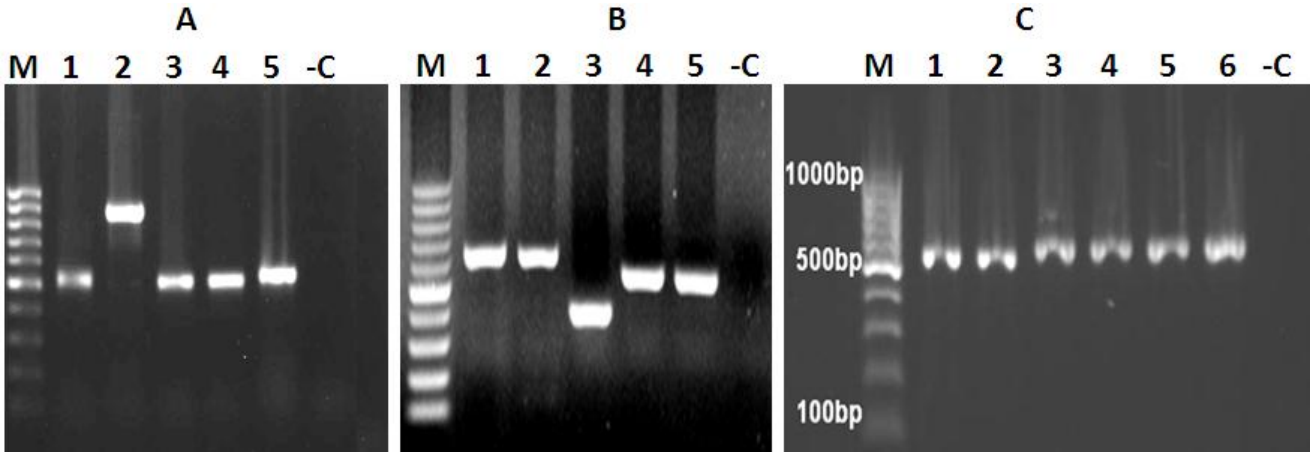

Figure 1. Representative gel electrophoresis of the ITS 1 and ITS 4 PCR products. (A) Lane 1: *Candida albicans* ATCC (~ 530 bp); lane 2: *C. glabrata* ATCC (~ 870 bp); lane 3: *C. Parapsilosis* ATCC (~ 515 bp); lane 4: *C. tropicalis* ATCC (~ 520 bp), lane 5: *C. albicans* 2639 (~ 530 bp. (B) Lane 1: *Candida famata* ATCC (~ 625 bp); 2 guil: *C. guilliermondii* ATCC (~ 610 bp); lane 3: *C. lusitaniae* ATCC (~ 370 bp); lane 4: *C. metapsilosis* ATCC (~ 517 bp); lane 5: *C. orthopsilosis* ATCC (~ 513 bp). (C) Lane 1: *Cryptococcus neoformans* 001 (~ 555 bp), lane 2: *Cryptococcus humicolus* 002 (~ 550 bp); lane 3: *Aspergillus fumigatus*; lane 4: *Aspergillus flavus*; lane 5: *Aspergillus niger*; lane 6: *Aspergillus terreus* (600 to 610 bp), respectively; lane -C: negative control which contains sterile distilled water instead of DNA template; lane M: DNA molecular marker (Fermentas).

Table 3. The sequences, lengths and product lengths of the triplex PCR primers.

Primer	Sequence	Primer length	Product length (bp)
CAF	5'- TTGTGTTGCTACATCACCAAC -3'	21	110
CAR	5'- CCACCGTCAACTAAATAAAG -3'	20	
CPF	5'- TCGTCGTAACTTGAGCG -3'	17	271
CPR	5'- TGGACGATTGGCGGTAT -3'	17	
CTF	5'- CCCATACGATTTATGGAAT -3'	19	501
CTR	5'- CCATTGACACAAGCATTTAC -3'	20	

CAF: *Candida albicans* forward primer; CAR: *C. albicans* reverse primer; CPF: *C. parapsilosis* forward primer; CPR: *C. parapsilosis* reverse primer; CTF: *C. tropicalis* forward primer; CTR: *C. tropicalis* reverse primer.

the three most common *Candida* species isolated from clinical specimens namely, *C. albicans, C. parapsilosis* and *C. tropicalis*. The developed simplex and triplex PCR methods targeted the *PLB* gene as a novel target. In order to specifically amplify segments from the *PLB* gene of the selected *Candida* species, specific primers were designed. Three species-specific primer pairs for simplex and triplex PCRs were designed successfully based on the most unique sequences that were found within the *PLB* genes of the respective *Candida* species. Table 3 shows the sequences and lengths of the designed primers and the lengths of the products as well.

Specificity test of the simplex and triplex PCR primers

The *Candida* species-specific primers of the simplex PCR were tested in terms of specificity, using fungal ATCC strains. Thirteen *Candida* ATCC strains, 4 *Aspergillus* species ATCC strains and 2 *Cryptococcus* species clinical isolates were cultured, and their DNA extracted and used as a template for the PCR amplification.

Specificity test for *Candida albicans* specific primers

The results of the gel electrophoresis of the PCR products amplified using the *C. albicans* species-specific primers (Figure 2) showed that the primers had annealed specifically to the *C. albicans* DNA, generating a PCR product that is around 1110 bp in length (confirmed by sequencing of the PCR products). There was no non-specific priming of DNA from other *Candida* species or fungal species at the annealing temperature of 60°C.

Specificity test for *Candida parapsilosis* specific primers

The annealing temperature of the *C. parapsilosis* specific

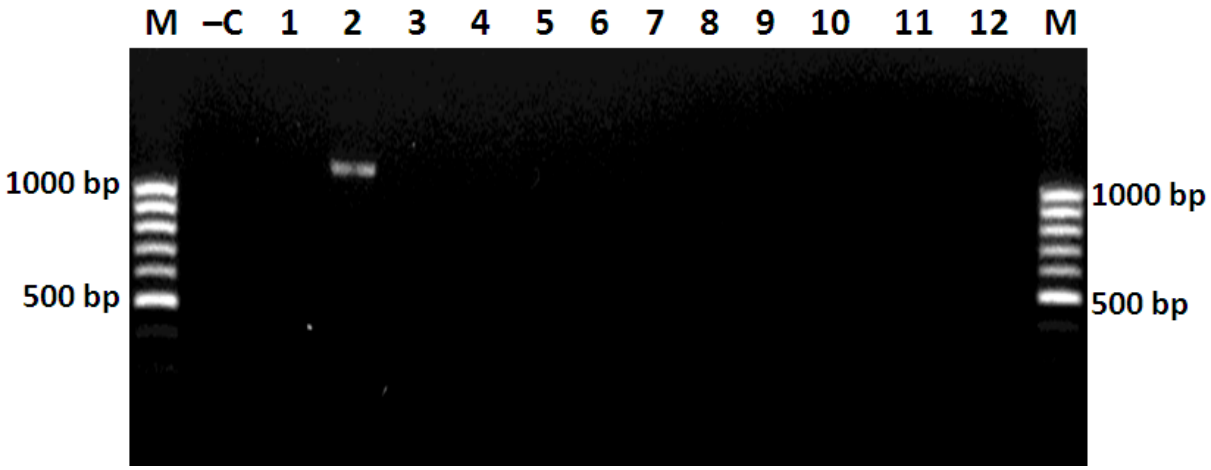

Figure 2. Representative gel electrophoresis of the simplex PCR using *Candida albicans* specific primers with DNA of *C. albicans* and other ATCC reference strains. Lane 1: *Aspergillus fumigatus*; lane 2: *C. albicans*; lane 3: *C. dubliniensis*, lane 3: *C. glabrata*; lane 4: *C. guilliermondii*; lane 5: *C. krusei*, lane 6: *C. lusitaniae*; lane 7: *C. metapsilosis*, lane 8: *C. parapsilosis;* lane 9: *C. orthopsilosis;* lane 10: *C. tropicalis*; lane 11: *Cryptococcus neoformans* (clinical strain); lane 12: *Cryptococcus humicolus* (clinical strain); lane -C: negative control which contains sterile distilled water instead of DNA template; lane M: DNA molecular marker. *Expected product sizes for *C. albicans* specific primers of the simplex PCR is 1110 bp, and as shown in Table 3.

primers at 55°C had amplified PCR product with 271 bp length (confirmed by sequencing of the PCR products). An expected band size was observed on the ethidium bromide-stained agarose gel under UV light (Figure 3) indicating that these primers had annealed to their targeted complimentary sequences, and there was no non-specific priming of DNA from other *Candida* species or even other fungal species at the annealing temperature of 55°C.

Specificity test for *Candida tropicalis* specific primers

In Figure 4, an expected band of 501 bp was observed on the ethidium-bromide-stained gel under UV light. This result indicated that the *C. tropicalis* species-specific primers had successfully annealed to their targeted region, resulting in the amplification of the targeted *PLB* gene region (confirmed by sequencing of the PCR products). There was no non-specific priming of DNA from other *Candida* species or fungal species at the annealing temperature of 53.4°C.

Specificity test for the triplex PCR species-specific primers

A triplex PCR was run using the three *Candida* species-specific primer sets that were designed for the amplification of *C. albicans, C. parapsilosis* and *C. tropicalis*. Our results showed that the designed primers were able to anneal to their targeted site on the DNA of the corresponding *Candida* species, and had allowed the amplification of the targeted sequences subsequently generating the expected PCR products of each primer pair. There was no non-specific priming of DNA from other *Candida* species or fungal species, at the annealing temperature of 50°C (Figure 5). The results showed that when the DNA samples from three *Candida* species were mixed together in one tube and subjected to multiplex PCR, using all 3 pairs of designed multiplex PCR primers, all 3 primer sets were able to amplify the targeted amplicons in a single PCR tube. Similarly, the reaction also worked for duplex.

DISCUSSION

Diagnosis of candidiasis is sometimes difficult because, no completely specific immunologic procedures for the identification of *Candida* currently exist (Willey et al., 2008). The detection of microbial DNA by PCR is without doubt one of the most powerful and popular tools for the early diagnosis and identification of human pathogens, including *Candida* species (White et al., 2009). The PCR methods described in the current study, which are simplex and triplex PCRs, have offered all the required characteristics of an appropriate molecular detection method: they are simple to conduct, rapid in identification of *Candida* species, specific in detection, and the results are highly reproducible. The “presence or absence of the band” identification system has eased the process of analyzing the result by only observing the ethidium bromide stained agarose gel under the exposure of UV light.

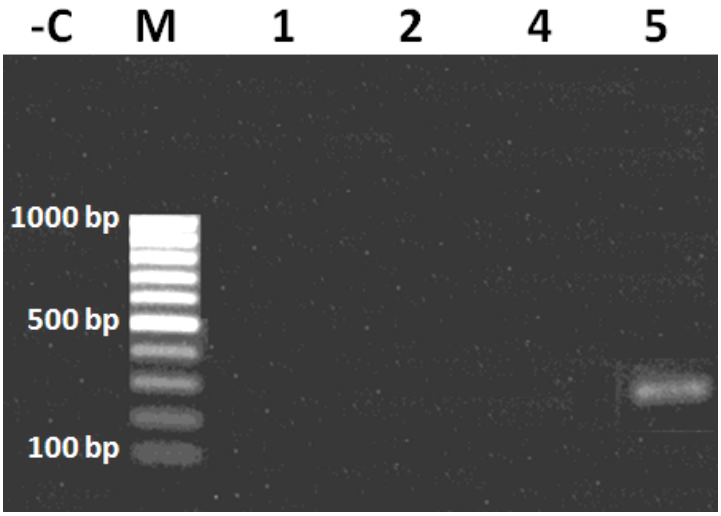

Figure 3. Representative gel electrophoresis of the simplex PCR using *Candida parapsilosis* specific primers with DNA of *C. parapsilosis* and other ATCC reference strains. Lane 1: *Candida albicans*; lane 2: *C. glabrata*; lane 3: *C. krusei*; lane 4: *C. tropicalis*; lane 5: *C. parapsilosis;* lane -C: negative control which contains sterile distilled water instead of DNA template; lane M: DNA molecular marker. *Expected product sizes for *C. parapsilosis* specific primers of the simplex PCR is 271 bp, and as shown in Table 3.

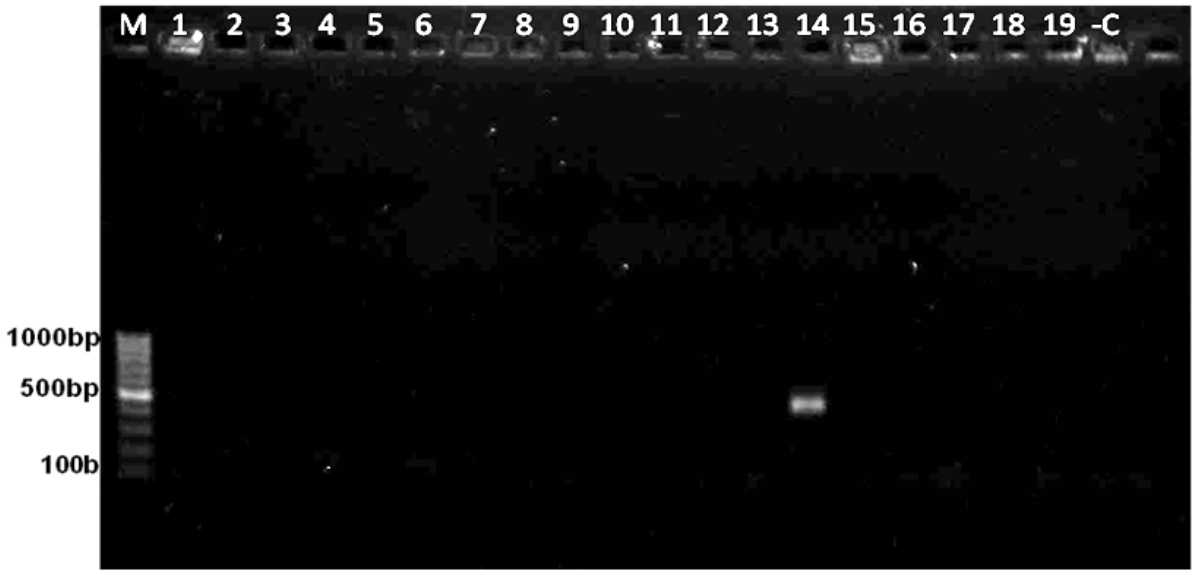

Figure 4. Representative gel electrophoresis of the simplex PCR using *Candida tropicalis* specific primers with DNA of *C. tropicalis* and other ATCC reference strains. Lane 1: *Candida albicans*; lane 2: *C. dubliniensis*; lane 3: *C. famata*; lane 4: *C. guilliermondii*; lane 5: *C. glabrata*; lane 6: *C. kefyr*; lane 7: *C. krusei*; lane 8: *C. lusitaniae*; lane 9: *C. metapsilosis*; lane 10: *C. parapsilosis;* lane 11: *C. orthopsilosi;* lane 12: *C. rugosa*; lane 13: *Aspergillus fumigatus*; lane 14: *C. tropicalis*; lane 15: *A. niger*; lane 16: *A. terreus*; lane 17: *A. flavus*; lane 18: *Cryptococcus neoformans* (clinical strain); lane 19: *Cryptococcus humicolus* (clinical strain); lane -C: negative control which contains sterile distilled water instead of DNA template; lane M: DNA molecular marker. *Expected product sizes for *C. tropicalis* specific primers of the simplex PCR is 501 bp, and as shown in Table 3.

The *PLB* gene of *Candida* species was chosen as the target site of designing *Candida* species-specific primers as a novel target which shows high variability in sequence among *Candida* species. This provided many

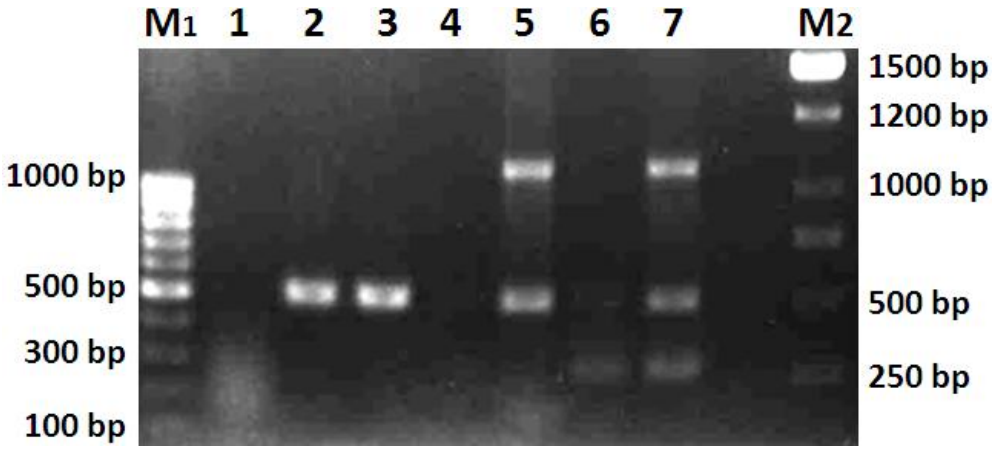

Figure 5. Representative gel electrophoresis of the triplex PCR which was designed to detect *C. albicans, C. parapsilosis,* and *C. tropicalis*. Lane 1: negative control which contains sterile distilled water instead of DNA template; lanes 2 and 3: DNA samples of *C. tropicalis*; lane 4: DNA samples of *C. glabrata*; lane 5: mixed DNA samples of two *Candida* species (*C. albicans* and *C. tropicalis*); lane 6: DNA sample of *C. parapsilosis*; lane 7: mixed DNA samples of three *Candida* species (*C. albicans, C. parapsilosis* and *C. tropicalis*) in one single tube; lane M1: 100 bp DNA ladder (Fermentas) and lane M2: 1 Kb ladder DNA molecular markers (Fermentas). *Expected product sizes for each primer pair are shown in Table 3.

possible unique sequences for each *Candida* species which enabled the designing of excellent species-specific primers that targeted the unique sequences of the *PLB* gene.

Additionally, the high variability in the *PLB* gene among *Candida* species allowed the designing of species-specific primers that produce distinctive product size for each *Candida* species which are suitable for the multiplex PCR. Consequently, these had allowed the specific detection of *Candida* species, since the designed species-specific primers for both simplex and triplex PCRs are only able to prime to their targeted *Candida* DNA, subsequently enabling the amplification of PCR products. Interestingly, the developed triplex PCR method in the current study, which targeted the *PLB* gene as a novel target, was able to simultaneously detect the three most commonly isolated *Candida* species such as *C. albicans*, *C. parapsilosis* and *C. tropicalis* from the clinical specimens in a single reaction tube, in a single PCR run. In order to confirm the specificity of the *Candida* species-specific primers, the experiments were repeated at least three times, using the extracted *Candida* DNA and other fungal DNA to confirm the reproducibility of the results. The species-specific primers were found to be specific to their targeted *Candida* DNA with no cross-priming to DNA from other *Candida* species or other fungal species in all the experiments using ATCC strains.

Previously, *C. albicans* and *Candida dubliniensis* could not be differentiated because of their nearly identical amplicon sizes (Fujita et al., 2001). The present study clearly discriminates between the targeted *Candida* species with at least 100 bp differences among the PCR products between each two different *Candida* species by both simplex and triplex PCR. More recently, a real-time multiplex tandem PCR assay was developed and evaluated for the detection and identification of fungi directly from blood culture specimens that have been flagged as positive (Lau et al., 2008, 2010). Undoubtedly, the real-time PCR has an advantage over the conventional PCR in terms of sensitivity. However, currently, the application of real-time PCR based methods in clinical microbiology laboratories are adopted exclusively for life-threatening viral infections such as human immunodeficiency virus (HIV), hepatitis and Influenza viruses, and difficult or non-cultivable bacterial pathogens. In addition, it is considered expensive when compared to the conventional diagnostic methods. Thus, the development of inexpensive DNA-based molecular techniques like the simplex and triplex PCRs of the current study may enhance the applications of the molecular methods as routine diagnostic tools in the clinical microbiology laboratories for identification and detection of pathogenic *Candida* species. Indeed, the newly developed methods, namely simplex and triplex PCRs, using species-specific primers targeting the *PLB* gene for differentiation of *Candida* species in this study, have provided desirable results, in terms of simplicity and specificity. Both methods are easy to conduct and generate diagnostic result within one working day. Since only few reagents are involved in triplex PCR and simultaneous differentiation of *Candida* species in a single reaction tube and one PCR run, this detection method costs less, compared to the simplex PCR. However, the advantage of the simplex PCR lies in its flexibility of adding more newly developed species-specific primers covering other

medically important human fungal pathogens, making it a useful molecular tool for rapid identification of a wide range of pathogenic fungal organisms at the species level.

The current result has persuaded us to pursue a new challenge in the development of molecular methods for the differentiation and identification of not only *Candida* species, but other medically important human fungal pathogens. Application of the simplex and triplex PCRs should be further evaluated in terms of the detection efficiency of *Candida* species from human blood samples, especially from patients who are under treatment with antifungal drugs.

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