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Application of LATE-PCR to Detect *Candida* and *Aspergillus* Fungal Pathogens by a DNA Hybridization Assay

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Asymmetric PCR preferentially amplifies one DNA strand for use in DNA hybridization studies. Linear-After-The-Exponential-PCR (LATE-PCR) is an advanced asymmetric PCR method which uses innovatively designed primers at different concentrations. This study aimed to optimise LATE-PCR parameters to produce single-stranded DNA of *Candida* spp. and *Aspergillus* spp. for detection via probe hybridisation. The internal transcribed spacer (ITS) region was used to design limiting primer and excess primer for LATE-PCR. Primer annealing and melting temperature, difference of melting temperature between limiting and excess primer and concentration of primers were optimized. In order to confirm the presence of single-stranded DNA, the LATE-PCR product was hybridised with digoxigenin labeled complementary oligonucleotide probe specific for each fungal genus and detected using anti-digoxigenin antibody by dot blotting. Important parameters that determine the production of single-stranded DNA in a LATE-PCR reaction are difference of melting temperature between the limiting and excess primer of at least 5℃ and primer concentration ratio of excess primer to limiting primer at 20:1. LATE-PCR products of *Candida albicans*, *Candida parapsilosis*, *Candida tropicalis* and *Aspergillus terreus* at up to 1:100 dilution and after 1 h hybridization time, successfully hybridised to respective oligonucleotide probes with no cross reactivity observed between each fungal genus probe and non-target products. For *Aspergillus fumigatus*, LATE-PCR products were detected at 1:10 dilution and after overnight hybridisation. These results indicate high detection sensitivity for single-stranded DNA produced by LATE-PCR. In conclusion, this advancement of PCR may be utilised to detect fungal pathogens which can aid the diagnosis of invasive fungal disease.

Keywords: **Fungal pathogens, LATE-PCR, hybridisation assay**

Introduction

The rise of invasive fungal infection (IFI) as a critical medical problem is related to the increase of immunocompromised patients [1]. IFI is one of the major causes of morbidity and mortality in immunocompromised patients [2]. The European Union for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative (EORTC) and the National Institute of Allergy and Infectious Diseases Mycoses Study (MSG) proposed that IFI can be defined as the existence of fungal matter either as mould or yeast in deep biopsy tissues or in nee-

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dle aspirates confirmed by culture and histo-pathological examination [2–4].

Aspergillus spp. and *Candida* spp. are the main fungal pathogens recovered from immunocompromised patients. Invasive aspergillosis and invasive candidiasis are two most prevalent non-endemic IFIs [2, 5]. Patients susceptible to invasive aspergillosis are those with predisposing risks such as structural lung disease or defects in host immune responses [6, 7], patients undergoing allogeneic haematopoietic stem cell transplantation [8], recipients of solid organ transplants [9], those with acute myelogenous leukaemia [10] or myelodysplastic syndrome [11] and those with other conditions of immunosuppression [12]. Occurrence of invasive candidiasis depends on the presence of a variety of risk factors such as haematological malignancy, neutropenia [2], recent abdominal surgery, use of central venous catheters, use of broad spectrum antibiotics [13, 14], long duration of hospitalization [15], total parenteral nutrition [16] and severe sepsis in critically ill surgical patients [17].

Therefore, to reduce IFI-related morbidity and mortality, clinicians need to identify high-risk patients and start early preemptive therapy. Besides this, rapid pathogen identification by using fast, non-culture based, sensitive diagnostic techniques allows early, targeted antifungal therapy that can help to reduce mortality among immunocompromised patients [18]. PCR-based diagnosis of IFI has been utilised for the last three decades [19–22], including the detection and identification of major pathogens such as *Candida*, *Aspergillus* and *Fusarium* [23, 24].

Asymmetric PCR is a method used to generate single-stranded DNA [25]. Although asymmetric PCR has been used for direct sequencing of the fungal internal transcribed spacer (ITS) region for strain identification [26], traditional asymmetric PCR yields are unstable and extensive optimisation is needed to increase the production of specific single-stranded products and to reduce non-specific amplification [27]. Linear-After-The-Exponential phase PCR (LATE-PCR) is a technique akin to asymmetric PCR but uses ingenious primer design at different concentrations to generate stable yields with high productivity and specificity [28]. The aim of this study was to optimize the LATE-PCR parameters for amplification and production of single-stranded nucleotide targets of major invasive fungal pathogens, namely *Candida* spp. and *Aspergillus* spp. that can be detected by oligonucleotide probes through a dot blot hybridisation assay.

Materials and Methods

Fungal Isolates & DNA Extraction

Fungal isolates for this research consisted of *Aspergillus fumigatus*, *Aspergillus terreus*, *Candida albicans*, *Candida tropicalis* and *Candida parapsilosis*. ATCC (American Type Culture Collection) strains of *C. parapsilosis* (ATCC 22019) and *C. albicans* (ATCC 10231) were provided by Novel Antibiotic Research Laboratory, UKM. Clinical isolates of *C. tropicalis*, *A. fumigatus* and *A. terreus* were provided by Mycology Unit, UKMMC (Universiti Kebangsaan Malaysia Medical Centre). The fungal colonies were homogenised mechanically using the BioMasher-II (OPTIMA, Japan) followed by further extraction steps using the DNeasy Plant Mini kit (Qiagen, USA) as recommended by the manufacturer. Fungal DNA was stored at -20℃.

Primer Design and Selection

The primer sequence which was used for this research is from the region of conserved ribosomal DNA sequences, known as ITS, allowing detection of fungal kingdom [29–32]. The universal primer sequences designed by White *et al.* [29] are ITS 1 (5'-TCC GTA GGT GAA CCT GCG G-3') which is the forward primer that amplifies at the end of rDNA 18S sequence and ITS 4 (5'-TCC TCC GCT TAT TGA TAT GC-3') which is the reverse primer that amplifies from the start of rDNA 28S sequence. In this experiment, the forward primer acts as the excess primer whereas the reverse primer acts as the limiting primer. Primers that were originally designed for conventional PCR were modified to match the LATE-PCR criteria for the generation of single stranded fungal DNA. The excess primer was shortened by the removal of 2 nucleotides from 5' end to decrease the melting temperature without affecting the target sequence that needs to be amplified. However the reverse primer chosen as the limiting primer was extended with the addition of either 12 or 10 nucleotides at 3' end to increase the melting temperature. In this study, SN1 ITS 1 (5'-CGT AGG TGA ACC TGC GG-3') was the excess primer, whereas EXN2 ITS 4 (5'-TCC TCC GCT TAT TGA TAT

Table 1. Primers used in LATE-PCR.

Primer	Primer sequence (5′-3′)	Melting temperature	Primer length
Excess primer, SN1 ITS 1	CGTAGGTGAACCTGCGG	55.8℃	17 nt
Limiting primer, EXN2 ITS 4	TCCTCCGCTTATTGATATGCTTAAGTTCAGCG	61.3℃	32 nt
Limiting primer, mEX1 ITS 4	TCCTCCGCTTATTGATATGCTTAAGTTCAG	58.2℃	30 nt

nt: nucleotides

GCT TAA GTT CAG CG-3') and EX1 ITS 4 (5'-TCC TCC GCT TAT TGA TAT GCT TAA GTT CAG-3') were the two limiting primers that were evaluated. OligoAnalyzer 3.1 (http://sg.idtdna.com/calc/analyzer) was used to analyse the melting temperatures, GC contents, molecular weights and length of the primers (Table 1). The primers were synthesized by IDT, Singapore (http://sg.idtdna.com/).

LATE-PCR

The LATE-PCR reaction included 1 μM excess primer, 0.05 μM limiting primer (concentration ratio of excess primer to limiting primer used was 20:1), 0.2 mM of each dNTP (Invitrogen, USA), 3 mM $MgCl_2$ (Invitrogen, USA), 0.04 units/μl Platinum TaqDNA Polymerase, 1X Green PCR buffer [20 mMTris-HCl (pH 8.4)/50 mMKCl] (Invitrogen, USA), 10 ng of fungal genomic DNA and deionised water in 25 μlvolume. Hot-start was used to avoid mispriming before the initial denaturation step. LATE-PCR reactions were carried out on a thermal cycler (BIO-RAD T 100^{TM} Thermal Cycler, Germany). In this experiment, LATE-PCR consisted of 65 cycles. An initial denaturation step of 5 min at 95℃ was followed by 65 cycles of 95℃ for 5 seconds (denaturation), 57–59℃ for 15 seconds (annealing) and 72℃ for 15 seconds (extension). A final extension step was run for 10 min at 72℃ to ensure complete synthesis and extension of DNA strand.

Dot Blot Hybridisation

The LATE-PCR product containing single stranded DNA was hybridised with DIG labeled complementary probe specific for each fungal genus and detected using anti-DIG antibody provided by DIG Nucleic Acid Detection kit (Roche, Germany). The oligonucleotide probes were labelled by tailing their 5' ends with digoxigenin and were synthesized by IDT Singapore. Hybridisation was performed as specified by the DIG Nucleic Acid Detection kit (Roche, Germany). The amplified LATE-PCR products were dotted on nitrocellulose membrane (Invitrogen, USA) and baked at 80℃ for two hours. Serial dilution of each sample was carried out beforehand. Three LATE-PCR product concentrations were used (undiluted, 1:10 and 1:100 dilutions). The blot was then hybridised with respective DIG labelled oligonucleotide probes in phosphate-buffered saline. The hybridisation procedures were performed at a temperature range of 39–45℃ and with five different incubation periods (overnight, 5 h, 3 h, 1 h and 30 min). Washing and blocking steps were carried out as specified by the DIG Wash and Block Buffer set (Roche, Germany). Following the hybridisation, washing and blocking steps, the antibody solution was added to the membrane. The blots were subjected to detection using anti-digoxigenin antibody conjugated to alkaline phosphatase and a color substrate (NBT-BCIP). Colour development was visible between 30 min and 1 h after the start of detection step. Cross reactivity testing was also carried out between each fungal genus probe and non-target LATE-PCR products. A negative control (non-template LATE-PCR product) was included for each test performed.

Results

In this study, LATE-PCR products were observed in agarose gel electrophoresis as faint bands which were smaller in size than the usual double-stranded DNA product. To confirm that single stranded products were obtained, hybridization with complementary oligonucleotide probes was performed. A dot blot procedure was carried out to detect hybridisation between the DIG-labeled oligonucleotide probes with complementary target nucleic acid fragments of *Candida* spp. and *Aspergillus* spp. on nitrocellulose membrane. The formation of a blue-purple coloured dot on the nitrocellulose membrane indicates successful hybridisation of single-stranded DNA (LATE-PCR product) to DIG labelled target probes. A conventional PCR followed by dot blotting of

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PCR product was carried out using the same fungal DNA to ensure double stranded products do not hybridise to the probes. The results of the dot blot hybridisation with LATE-PCR products using the primer set of SN1 ITS 1 (forward primer/excess primer) and EXN2 ITS 4 (reverse primer/limiting primer) based on 5 different hybridisation incubation periods and three different concentrations of LATE-PCR product are shown in Figs. 1, 2.

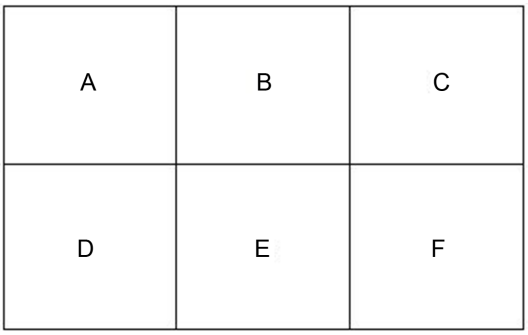

Fig. 1. Preparation of nitrocellulose membrane with divisions for dotting respective samples (A to F) before the hybridisation process. (A) Undiluted LATE-PCR product, (B) LATE-PCR product diluted to 1:10, (C) LATE-PCR product diluted to 1:100, (D) DIG Nucleic Acid (kit) positive control, (E) Negative control, (F) Non-target LATE-PCR product.

The minimum concentration of LATE-PCR products detected and the minimum hybridisation incubation period varies according to the fungal species. The minimum concentration of LATE-PCR product detected was at 1:100 dilution with hybridisation incubation period of 1 h for *C. albicans* (9.6 ng/µl), *C. parapsilosis* (8.5 ng/µl) and *C. tropicalis* (8 ng/µl) and hybridisation incubation period of 30 min for *A. terreus* (9.5 ng/µl). The LATE-PCR products of *C. albicans* (96 ng/µl), *C. parapsilosis* (85 ng/µl) and *C. tropicalis* (80 ng/µl) at 1:10 dilution hybridised to oligonucleotide probes after 30 min of hybridization. For *A. fumigatus*, the LATE-PCR product hybridised to target probe relatively late after 5 h incubation, with undiluted LATE-PCR product (770 ng/µl), while the detection limit is at 1:10 dilution of LATE-PCR product (77 ng/µl) with overnight hybridization period. The LATE-PCR product contains both single-stranded

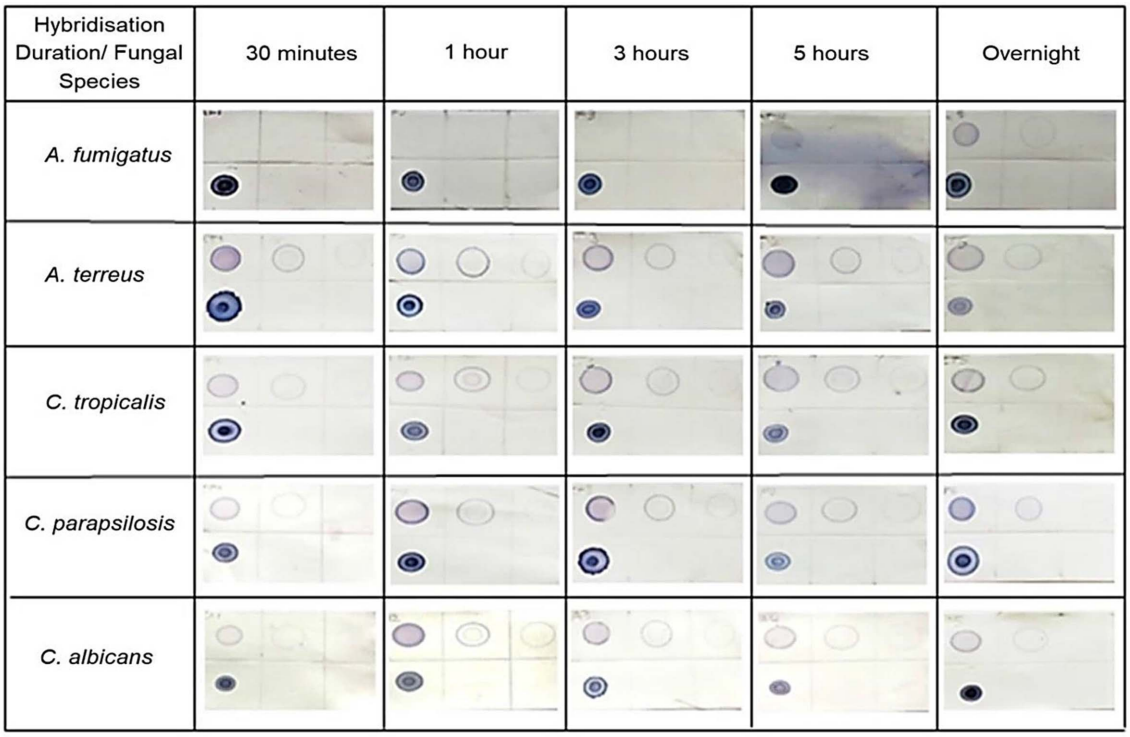

Fig. 2. LATE-PCR product detection by dot blot hybridisation with different species of fungi at different concentrations of LATE-PCR products and different incubation periods of hybridisation. Each membrane was prepared as shown in Fig. 1. After 1 h hybridisation, LATE-PCR products at 1:100 dilution were detectable for *A. terreus*, *C. tropicalis*, *C. parapsilosis* (less visible) and *C. albicans*.

DNA and double-stranded DNA. Therefore, it is important to note that the actual concentration of single-stranded LATE-PCR product needed to hybridise to the target oligonucleotide probes is lower than the concentrations stated above.

Discussion

This study was conducted to optimise the LATE-PCR parameters for the production of single-stranded nucleotides of *Candida* spp. and *Aspergillus* spp. which was then detected by oligonucleotide probes. The LATE-PCR parameters optimised were primer annealing temperature, melting temperature of limiting primer and excess primer, difference of melting temperature between excess primer and limiting primer and primer concentration ratio of excess primer to limiting primer. The presence of single-stranded product was detected with a DIG labelled oligonucleotide probe through dot blot hybridisation.

The length of a limiting primer should be approximately 24–32 nucleotides to achieve a high melting temperature for LATE-PCR [28]. Increasing the melting temperature of the limiting primer above the melting temperature of the excess primer boosts the efficiency and specificity to produce good quantities of single-stranded DNA. A true optimal annealing temperature for both primers can be used, one that is sufficiently low relative to the melting temperature of the limiting primer to allow adequate utilization of the limiting primer but high relative to the melting temperature of the excess primer to reduce amplification of non-specific products by the excess primer [27]. In this experiment, 2 primer pairs were tested which were SN1 ITS 1/EXN2 ITS 4 and SN1 ITS 1/EX1 ITS 4. The excess primer, SN1 ITS 1 with a length of 17 nucleotides has a melting temperature of 55.8℃, whereas the melting temperature of the limiting primers, EXN2 ITS 4 (32 nucleotides) and EX1 ITS 4 (30 nucleotides) are 61.3℃ and 58.2℃, respectively. The efficiency and specificity of optimal amplification is achieved when the melting temperature of limiting primer is approximately 5℃ higher than that of the excess primer [27]. In this study, the primer pair SN1 ITS 1/EXN2 ITS 4 showed better amplification and generation of single stranded DNA as the melting temperature of EXN2 ITS 4 was 5.5℃ higher than SN1 ITS 1. Darker coloured product was observed on the blots using primer pair SN1 ITS 1/EXN2 ITS 4.

LATE-PCR uses 2 primers at different concentrations. The concentration ratio of excess primer to limiting primer in this study was 20:1. The concentration ratio of excess primer to limiting primer must be within 20:1 to 40: 1 for efficient linear amplification [28]. Reducing the concentration of the limiting primer lowers its melting temperature to below the primer annealing temperature [33], however with the addition of nucleotides to extend the length of the limiting primer, the melting temperature of the limiting primer is increased [34] to at least as high as that of the excess primer or even higher as observed in this study.

In this experiment, a gradient of primer annealing temperature in the range of 57–59℃ was tested to ensure optimum annealing temperature that matches the LATE-PCR criteria. The optimal temperature that was set for the annealing step in LATE-PCR was 57.8℃, which was 3.5℃ lower than the melting temperature of the limiting primer. Annealing temperature of at least 2℃ below the melting temperature of the limiting primer generates single stranded DNA without the amplification of nonspecific product [27].

To determine the sensitivity of hybridisation between LATE-PCR product and target probe each LATE-PCR product was serially diluted up to 1:100 dilution and with reduction of incubation period of hybridisation from overnight to 30 min. The sensitivity of hybridisation was lower for *A. fumigatus* compared to other species. The sequence variation among *Aspergillus* species in ITS 2 region was minimal compared to ITS 1 [30, 35, 36]. In this study, for hybridization, a probe specific for the genus *Aspergillus* was selected from ITS 1 region, whereas a probe specific for *Candida* was selected from ITS 2 region. Thus, the selection of a probe specific for *Aspergillus* from ITS 2 region may increase the hybridisation rate for *A. fumigatus*. In a previous study, the species-specific probe for 7 species of *Aspergillus* was selected from ITS 2 region [34]. No cross-reaction occurred between the probe specific for *Aspergillus* and species of *Candida* (*C. albicans, C. parapsilosis* and *C. tropicalis*) and between the probe specific for *Candida* and species of *Aspergillus* (*A. terreus* and *A. fumigatus*).

The melting temperature of the oligonucleotide probes specific for *Aspergillus* and *Candida* were 50.9℃ and

47.2℃, respectively. The hybridisation temperature was set at a temperature lower than the melting temperature of the probes to assist the hybridization process between LATE-PCR product and probe. Although the hybridisation temperature was tested from 45℃ and reduced to 39℃, the result of hybridisation was approximately similar in that range of hybridisation temperature. Therefore, the lowest temperature, 39℃ was used as the optimum temperature for all hybridisation process. The use of temperature less than 39℃ did not produce satisfactory hybridisation result.

LATE-PCR successfully amplified and produced single strands of nucleotide targets of *Candida* spp. and *Aspergillus* spp. which were then identified with dot blot hybridisation with target oligonucleotide probes. The advancement of this PCR technique enables further development of rapid assays in the future such as biosensors that can detect specific single-stranded DNA products of pathogenic fungi including antifungal resistant species for the purpose of diagnosing invasive fungal diseases.

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Conflict of Interest

The authors declare that they have no conflict of interests.

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