UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF NOVEL PRIMERS AND PROBES BASED ON ITS 2 REGION USING NESTED-PCR AND DNA HYBRIDIZATION ARRAY FOR *CANDIDA* SPECIES IDENTIFICATION

DAVID CHIENG CHING SOO

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By

DAVID CHIENG CHING SOO

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

February 2007

Specially dedicated to,

My beloved grandmother, parent, sister, Tsuey Peng, and all my family members

For their invaluable love, understanding, encouragement and patience

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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Chairman: Chong Pei Pei, PhD

Faculty: Faculty of Medicine and Health Sciences

Sensitivity, specificity, simplicity, speed, and economy fairly well describe the desirable attributes of any clinical diagnostic test. In the mycology laboratory, the detection and differentiation of *Candida* species usually rely on the morphological, physiological, and biochemical characteristics which are complex, frequently slow to appear, sometimes vary within a species, and usually require significant experience to evaluate.

In this study, novel primers and probes had been developed for the identification of 8 *Candida* species using nested-PCR and DNA hybridization array by focusing on a

single parameter. This parameter is the identification of nucleotide sequence from ITS 2 region that is known to be unique for the *Candida* species. These distinctive sequences were used to design and develop *Candida* species-specific primers and probes for nested-PCR and DNA hybridization array, respectively. For nested-PCR, the universal fungal primers, ITS 3 and ITS 4 were used to amplify the portion of the 5.8S and 28S rDNA, and the ITS 2 region of *Candida* species. The amplicons were used as template together with developed species-specific primers in the subsequent amplification. The results were analyzed using agarose gel electrophoresis. In addition, species-specific probes were used to hybridize to the biotinylated *Candida* species ITS 2 amplicons via DNA hybridization array. The amplicons were detected colorimetrically in strip format.

The sensitivity and specificity of the *Candida* species-specific primers and probes were evaluated. The 8 *Candida* ATCC strains and 24 clinical isolates did not exhibit cross-priming and cross-hybridization with the species-specific primers and probes in both assays and all the *Candida* species were correctly identified. In simulated clinical specimens, the sensitivity of the nested-PCR for *Candida* species detection was 10 cells/mL. However, the detection limit for both PCR using species-specific primers and DNA hybridization array using species-specific probes for the detection of *Candida* culture DNA was 1-10 cells/mL.

Additionally, a preliminary study was done for the screening of 40 healthy donors' sera using the real-time PCR for the detection of *Candida albicans* using species-specific primers, CAL1 and CAL3. The melting curve was used for the analyses of the results. 3 out of 40 samples were found to be positive for *Candida albicans*. It is suggested that the real-time PCR may not be able to distinguish the individuals who are colonized from those who are infected.

In conclusion, the nested-PCR and DNA hybridization array using the developed species-specific primers and probes, respectively, in this study are robust, sensitive and can be easily integrated into a clinical diagnostic laboratory with the potential for same-day diagnosis of *Candida* infection. In addition, the simultaneous differentiation of *Candida* species via DNA hybridization array allows faster and simpler diagnosis compare to nested-PCR. For real-time PCR screening of the healthy donors' sera, further evaluation needs to be done to determine a threshold as standard guideline to detect the infectious and colonized *Candida albicans* and non-*albicans Candida* species.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PERKEMBANGAN PRIMER DAN PROBE YANG NOVEL BERDASARKAN BAHAGIAN ITS 2 MELALUI NESTED-PCR DAN DNA HYBRIDIZATION ARRAY UNTUK PENGESANAN SPESIES *CANDIDA*

Oleh

DAVID CHIENG CHING SOO

Februari 2007

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Sensitif, spesifik, ringkas, cepat, dan murah disifatkan sebagai ciri-ciri yang diingini bagi setiap ujian diagnostik di makmal. Dalam makmal mikologi, pengesanan dan pembezaaan bagi spesies *Candida* kebiasanya bergantung kepada penampilan sifat secara morfologi dan fisiologi serta ciri-ciri biokimia yang kompleks yang muncul lewat. Kadangkala ianya berbeza sesama spesies dan pengalaman pegawai bagi menilai keputusan amat diperlukan dalam diagnosis tersebut.

Dalam projek ini, primer-primer dan probe-probe baru telah dibangunkan bagi pembezaan antara 8 spesies *Candida* melalui dua kaedah molekul, iaitu

"nested-PCR" dan "DNA hybridization array" dengan menumpu kepada satu parameter tunggal. Parameter tersebut adalah pengesanan jujukan nukleotida daripada bahagian ITS 2 yang unik untuk spesies *Candida*. Jujukan nukleotida tersebut ini telah digunakan untuk mereka dan menghasilkan primer dan probe yang spesifik bagi spesies *Candida* untuk digunakan dalam "nested-PCR" dan "DNA hybridization array". Bagi "nested-PCR", primer fungi yang universal iaitu ITS 3 dan ITS 4 telah digunakan dalam amplifikasi sebahagian daripada 5.8S dan 28S rDNA, dan seluruh bahagian ITS 2 spesies *Candida*. Hasil PCR ini telah digunakan sebagai templat bersama dengan primer spesies *Candida* yang spesifik untuk amplifikasi berikutnya. Hasil amplifikasi tersebut dianalisis menerusi elektroforesis gel agarosa. Bagi "DNA hybridiazation array", probe spesies *Candida* yang spesifik digunakan dihibridkan dengan hasil amplifikasi bahagian ITS 2 spesies *Candida* yang dilabeldengan biotin. Ianya dikesan secara kolorimetri pada strip yang disediakan.

Sensitiviti dan kespesifikan primer dan probe yang spesifik kepada spesies *Candida* telah dinilai. 8 strain ATCC dan 24 sampel spesies *Candida* tidak menunjukkan sebarang "cross-priming" dan "cross-hybridization" terhadap primer dan probe yang diuji dalam kedua-dua kaedah tersebut. Semua sampel dan strain *Candida* telah dikenalpasti dengan tepat. Dalam spesimen-spesimen klinikal yang tersimulasi, sensitiviti bagi "nested-PCR" dalam pengesanan spesies *Candida* ialah 10 sel/ml. Walau bagaimanapun, had pengesanan bagi "PCR" dan "DNA hybridization array"

dengan menggunakan primer dan probe, masing-masing, dalam pengesanan DNA spesies *Candida* dari kultur ialah 1-10 sel/ml.

Sebagai tambahan, satu kajian awal telah dijalankan untuk penyaringan sampel-sampel serum daripada 40 penderma darah yang sihat melalui "real-time PCR" bagi pengesanan terhadap spesies *Candida albicans* menggunakan primer yang spesifik terhadap *Candida albicans,* iaitu *CAL 1* dan *CAL 3*. Hasilnya dianalisa menggunakan "melting curve". 3 daripada 40 sampel tersebut didapati positif untuk *Candida albicans*. Keputusan tersebut mencadangkan bahawa "real-time PCR" berkemungkinan tidak berupaya bagi membezakan individu yang mempunyai *Candida albicans* yang bersifat komensal daripada individu yang terjangkit.

Sebagai kesimpulan, "nested-PCR" dan "DNA hybridization array" yang digunakan dalam projek ini adalah sensitif dan spesifik dengan menggunakan primer dan probe yang spesies-spesifik masing-masing. Ianya amat berpotensi untuk tujuan diagnosis di dalam makmal klinikal kerana keputusan pengesanan spesies *Candida* boleh dihasilkan dalam masa satu hari. Di samping itu, pembezaaan spesies *Candida* secara setara melalui "DNA hybridization array" boleh menghasilkan keputusan diagnostik dengan cepat dan mudah. Bagi penyaringan sampel serum daripada penderma-penderma sihat melalui "real-time PCR", ianya perlu dilakukan terhadap sejumlah sampel yang besar bagi menentukan satu ambang sebagai garis panduan yang standard untuk mambezakan sama ada seseorang itu mempunyai *Candida*

albicans yang bersifat komensal atau terjangkit atau spesies-spesies *Candida* yang lain.

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I certify that an Examination Committee has met on 9 February 2007 to conduct the final examination of David Chieng Ching Soo on his Master of Science thesis entitled "Development of Novel Primers and Probes Based on ITS 2 Region Using Nested-PCR and DNA Hybridization Array for *Candida* Species Identification" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee are as follows:

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Date: 14 JUNE 2007

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

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DAVID CHIENG CHING SOO

Date: 4 APRIL 2007

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ATCC	American Type Culture Collection
AP	Alkaline phosphatase
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bps	Base pairs
CFU	Colony forming units
dNTP	Deoxynucleotide triphosphate
EDTA	Disodium ethylene-diaminetetra acetate.$2H_2O$
EIA or ELISA	Enzyme-linked Immunosorbent assay
EtBr	Ethidium bromide
ITS	Internal Transcribed Spacer
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
LiPA	Line probe assay
$MgCl_2$	Magnesium chloride
MW	Molecular weight
Na_2HPO_4	Disodium hydrogen phosphate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NBT	Nitro-blue-tetrazolium
NH_4SO_4	Ammonium sulfate
n-PCR	Nested-PCR