UNIVERSITI PUTRA MALAYSIA

MOLECULAR TECHNIQUES FOR DETECTION OF *CANDIDA* SPECIES

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By

NABIL SAAD MOHAMMED HARMAL

**Thesis Submitted to the School of Graduate Studies,
Universiti Putra Malaysia in Fulfillment of the Requirement for the
Degree of Doctor of Philosophy**

September 2011

DEDICATION

To my late mother Ateqah.

To my father for his unselfish support and love that you have provided me during my life.

To my wife Eman for her affection and constant support.

To my children Abdul-Rahman, and Ghufran for being a great source of motivation, strength and laughter.

To my sisters, brothers and family in law for their encouragement and moral assistance.

Abstract of thesis presented to the senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

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Chairman: Associate professor. Chong Pei Pei, PhD

Faculty : Medicine and Health Sciences

Candida species are a major cause of invasive infections, in both critically ill and immunocompromised patients. Thus, rapid identification of these pathogens may facilitate specific therapy and patient management. The development of rapid and specific diagnostic methods remains a challenge. Different methods for the detection of *Candida* species including simplex and multiplex PCRs, PCR/RLB (Reverse Line Blot) hybridization technique, and finally a novel pan-fungal (yeast) specific primers for the detection of medically important *Candida* species and other fungal yeasts have been developed in this study. ATCC strains of *Candida* species (n=13), *Aspergillus* species (n=4), *Pichia* species (n=1), and two clinical isolates of *Cryptococcus* species were used in this study to confirm the specificity of the designed primers. For evaluation of the developed simplex and multiplex PCRs and PCR/RLB, 23 clinical strains of the following *Candida* species; *C. albicans* (n=5), *C. glabrata* (n=5), *C. parapsilosis* (n=5),

and *C. tropicalis* (n=8) obtained from University Malaya Medical Centre (UMMC) between 2003 and 2004 were used (Table 3.2). In addition, 44 blood samples taken from BacT/Alert blood culture bottles were obtained from UMMC during the period from December 2009 to August 2010 were used for further validation.

Simplex and multiplex PCR methods were developed for the detection of the four most common *Candida* species isolated from clinical samples: *C. albicans*, *C. glabrata*, *C. parapsilosis*, and *C. tropicalis*. The developed methods target the phospholipase B gene (*PLB*) as a novel target. The alignment software surprisingly showed that this gene contain large sequence variation among *Candida* species. The nucleotide sequence variability between *C. tropicalis* and *C. glabrata* was as high as 75%. Four pairs of species-specific primers were designed for the corresponding *Candida* species and used in the simplex PCR. New primer sets were designed for multiplexing purpose for *C. albicans*, *C. parapsilosis*, and forward primer for *C. glabrata*. While the reverse primer of *C. glabrata* and primer site of *C. tropicalis* used in the simplex PCR were also suitable for the multiplex PCR. On the other hand, novel species-specific primers and probes were designed for the development of specific PCR and PCR/RLB hybridization techniques for the detection of the less commonly isolated *Candida* species, namely; (*C. famata, C. guilliermondii, C. lusitaniae, C. metapsilosis, and C. orthopsilosis*). The methods developed targeted the internal transcribed spacer (ITS) region. The validity tests showed that the designed primers and probes achieved highly specific identification of the selected species, with no cross- reaction with the other tested fungal

species. The methods were able to detect <10 cell/ml of culture broth when tested repeatedly for the corresponding *Candida* species.

Finally a novel set of pan-fungal (yeasts) specific primers for the detection of medically important *Candida* species and other fungal yeasts were developed, by first amplifying DNA from *Candida* species, and other yeasts using the random amplified polymorphic DNA (RAPD) technique with arbitrary primers. A 550-bp PCR product, amplified with primers OPA-03 and NS-2 and common to *Candida* species and the other yeast, was selected for further evaluation. The fragment was cloned and subsequently sequenced. Based on the sequence data, two primers were selected and synthesized. The primer pair amplified a single fragment of 340 bp from DNA of *Candida* species and the other yeast. No amplification product was detected in DNA from *Aspergillus* species, bacteria, and human. This assay thus allows the precise and rapid detection of *Candida* species and other yeasts like *Cryptococcus* species.

In conclusion, the results showed that the developed methods could be used for the rapid, specific and sensitive detection of both the most and less commonly encountered *Candida* species from the clinical specimens using the simplex/multiplex PCRs and PCR/RLB hybridization techniques, respectively. In addition, the pan-*Candida*/yeasts PCR developed in this study enables wide range detection of *Candida* species and other medically important yeasts like *Cryptococcus* species.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai keperluan untuk ijazah Doktor Falsafah

TEKNIK PENGECAMAN MOLEKUL *CANDIDA* SPESIES

Oleh

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Pengerusi : Profesor Madya Chong Pei Pei, PhD
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Spesies *Candida* adalah punca utama merebaknya jangkitan kulat di kalangan pesakit kritikal dan pesakit yang mempunyai sistem ketahanan imun yang lemah. Oleh yang demikian, identifikasi pantas patogen ini dapat memudahkan terapi khusus dan pengurusan pesakit. Namun begitu, kaedah diagnostik yang cepat dan khusus tetap menjadi cabaran buat para saintis. Dalam kajian ini, pelbagai kaedah untuk mengesan spesis *Candida* telah dikaji umpamanya simplex dan multipleks PCR, teknik hibridisasi PCR / RLB (Reverse Line Blot) serta inovasi primer pan-fungi (sejenis kulat) khusus untuk pengecaman spesis *Candida* dan kulat-kulat lain yang penting dalam bidang perubatan. ATCC jenis spesies *Candida* (n = 13), spesies *Aspergillus* (n = 4), spesies *Pichia* (n = 1) serta dua isolat klinikal spesies *Cryptococcus* telah digunakan dalam kajian ini untuk menguji dan mengesahkan kespesifikan primer. Untuk penilaian simplex dikembangkan dan PCR multipleks dan PCR/RLB, 23 strain klinikal spesies

Candida berikut; *C. albicans* (n = 5), *C. glabrata* (n = 5), *C. parapsilosis* (n = 5), dan *C. tropicalis* (n = 8) yang diperolehi dari University Malaya Medical Centre (UMMC) antara 2003 dan 2004 digunakan (Jadual 3.2). Selain itu, 44 sampel darah diambil daripada botol kultur Bact/Alert yang diperolehi dari UMMC pada Disember 2009 sehingga Ogos 2010 dan divalidasi untuk kajian yang selanjutnya.

Kaedah simpleks dan multipleks PCR telah dicipta untuk mengesan empat isolat klinikal spesies Candida yang penting seperti: *C. albicans*, *C. glabrata*, *C. parapsilosis*, dan *C. tropicalis*. Kaedah yang dicipta ini adalah khusus untuk mengesan gen B fosfolipase (*PLB*) sebagai sasaran baharu. Peranti perisian alignment menunjukkan bahawa gen ini mengandungi variasi jujukan yang besar di antara spesies *Candida*. Jujukan variasi nukleotida di antara *C. tropicalis* dan *C. glabrata* mencecah 75%. Empat pasangan primer spesies-spesifik telah direka untuk *Candida* spesies dan digunakan dalam PCR simplex. Beberapa set primer telah direka bagi tujuan multiplexing *C. albicans, C. parapsilosis*, dan forward primer untuk *C. glabrata*. Reverse primer bagi *C. glabrata* dan bahagian primer *C. tropicalis* primer yang digunakan dalam PCR simpleks juga sesuai untuk PCR multipleks. Pada masa yang sama, primer dan probe yang spesies-spesifik telah direka untuk inovasi PCR yang spesifik serta teknik hibridisasi PCR/RLB bagi mengesan *Candida* spesies yang jarang ditemui seperti; (*C. famata, C. guilliermondii, C. lusitaniae, C. metapsilosis,* dan *C. orthopsilosis*). Kaedah yang direka mensasarkan pada bahagian spacer dalaman transkripsi (ITS). Ujian pengesahan menunjukkan bahawa primer dan probe yang direka mencapai identifikasi yang sangat spesifik. tanpa reaksi bersilang dengan spesies kulat

yang lain. Kaedah ini mampu mengesan <10 sel/ml pati kultur apabila diuji berulang kali untuk mendapatkan korespondensi spesies *Candida*.

Seterusnya, primer khusus pan-fungi (yis) telah dibangunkan untuk pengecaman spesies *Candida* yang mempunyai kepentingan perubatan dan yis fungi yang lain, menerusi amplifikasi pertama DNA daripada spesies *Candida*, dan menggunakan teknik amplifikasi polimorfik DNA rawak (RAPD) bersama primer yang dipilih secara rawak. Produk 550-bp PCR telah dipilih untuk penilaian lebih lanjut dengan mengamplifikasikannya bersama primer OPA-03 dan NS-2 yang biasa digunakan untuk spesies *Candida* dan yis yang lain. Bahagian yang telah diamplifikasikan ini kemudiannya diklon dan dijujukkan. Berdasarkan jujukan data, dua primer telah dipilih dan disentesis. Pasangan primer itu mengamplifikasi fragmen sebanyak 340 bp daripada DNA spesies *Candida* dan yis yang lain. Tiada amplifikasi dikesan dalam DNA spesies *Aspergillus*, bakteria dan manusia. Namun ujian ini membolehkan pengecaman yang tepat dan cepat bagi spesies *Candida* dan yis yang lain seperti spesies *Cryptococcus*.

Kesimpulannya, hasil kajian menunjukkan bahawa kaedah yang dibangunkan boleh digunakan untuk pengecaman secara cepat, khusus dan sensitif bagi kedua-dua spesies *Candida* yang paling dan jarang ditemui pada spesimen klinikal, masing-masing dengan menggunakan teknik PCRs simpleks/multipleks dan hibridisasi PCR/RLB. Selain itu, pan-*Candida*/yeast-*Candida* PCR yang dibangunkan dalam kajian ini membolehkan pengecaman pelbagai spesies *Candida* dan yis lain yang mempunyai kepentingan perubatan seperti spesies *Cryptococcus*.

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I certify that a Thesis Examination Committee has met on 7th September 2011 to conduct the final examination of Nabil Saad Mohammed Harmal on his thesis entitled "Development of Molecular Techniques for Detection of *Candida* Species" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

Members of the Thesis Examination Committee were as follows:

Noritah Omar, PhD
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Date:

This thesis was submitted to the senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of **Doctor of Philosophy**. The members of the Supervisory Committee were as follows:

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Date:

DECLARATION

I declare that the thesis is my original work except for quotations and citation which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or other institutions.

NABIL SAAD MOHAMMED HARMAL

Date: 7 September 2011

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