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IDENTIFICATION OF PROTEIN BIOMARKERS FOR CANDIDA PARAPSILOSIS AND CANDIDA TROPICALIS

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IDENTIFICATION OF PROTEIN BIOMARKERS FOR *CANDIDA PARAPSILOSIS* AND *CANDIDA TROPICALIS*

By

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IDENTIFICATION OF PROTEIN BIOMARKERS FOR *CANDIDA PARAPSILOSIS* AND *CANDIDA TROPICALIS*

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June 2014

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Candida species are the major human fungal pathogens and incidence of systemic candidiasis has been rising over the years with *Candida albicans* as the main species isolated. However, *Candida parapsilosis* and *Candida tropicalis* have emerged recently as increasingly prevalent pathogens, but only few studies have focused on them thus far. In the first part of this study, systemic infection of *C. parapsilosis* and *C. tropicalis* were generated in mice via intravenous challenge and their pathogenicity was studied. It was demonstrated that mice challenged with *C. parapsilosis* and *C. tropicalis* exhibited different survival rate, with death only observed for *C. tropicalis*-infected mice. Besides, *C. tropicalis*-infected mice displayed higher fungal tissue burden and more severe kidney damage. Overall, the results indicate that *C. tropicalis* was more virulent than *C. parapsilosis* and suggests that specific virulence factors such as morphogenesis may account for variation in pathogenesis. In another context, difficulty in establishing definitive diagnosis for candidasis has prompted the search of biomarkers for the disease. Squalene synthase is a novel antigenic protein of *C. tropicalis* that was discovered from a previous study. To investigate its potential as a biomarker candidate, this protein was expressed in *Pichia pastoris* and the fusion protein was purified by affinity chromatography. The results showed that the purified recombinant protein was specifically recognized by polyclonal antibodies from *C. tropicalis*-infected mice on Western blot, suggesting that the protein could be a potential biomarker for *C. tropicalis*. However, further testing is needed to confirm its utility. To further discover protein biomarkers for *C. parapsilosis* and *C. tropicalis* and to understand their host-pathogen interactions, an immunoproteomic analysis was performed. For this purpose, cell wall proteins-enriched fractions of *C. parapsilosis* and *C. tropicalis* were systemically screened for antigens using antisera obtained from experimentally infected mice. This analysis led to the identification of 12 immunogenic proteins each for *C. parapsilosis* and *C. tropicalis*, of which 8 were common antigens for both species. Among these antigens, 14 have been previously reported as antigens of *C. albicans*, whereas isocitrate dehydrogenase (Idh2p) and dihydrolipoyllysine-residue

succinyltransferase (Kgd2p) were novel immunogenic proteins that were reported for the first time for *Candida* species. The present work showed that these antigens were expressed *in vivo* during infection and are likely to play important roles in pathogenesis. Next, the newly reported antigens, Idh2p and Kgd2p were overexpressed as recombinant proteins in *Escherichia coli* and subsequently purified by affinity chromatography. The antigenicity of the recombinant proteins was verified by immunoblotting using antisera from infected mice. This preliminary work suggests that the two proteins may find potential application as biomarker for *C. parapsilosis* and *C. tropicalis*. However, additional work is required to evaluate the usefulness of these proteins. Collectively, findings from the mouse model of infection and antigen profiling by immunoproteomics help to improve understanding on host response to *C. parapsilosis* and *C. tropicalis* infection, as well as discovering new protein antigens to be employed as disease biomarker candidates. This work also described the production of several antigenic recombinant proteins that lays the foundation for further research.

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

PENGENALPASTIAN PENANDA BIOLOGI PROTEIN UNTUK *CANDIDA PARAPSILOSIS* DAN *CANDIDA TROPICALIS*

Oleh

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Jun 2014

Pengerusi: Profesor Madya Chong Pei Pei, PhD
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Spesies *Candida* merupakan kulat patogen utama untuk manusia dan kejadian candidiasis sistemik telah semakin meningkat sejak beberapa tahun kebelakangan ini dengan *Candida albicans* sebagai spesies utama. Walau bagaimanapun, *Candida parapsilosis* dan *Candida tropicalis* telah muncul baru-baru ini sebagai patogen kulat yang semakin berleluasa tetapi sehingga kini hanya sedikit kajian sahaja yang tertumpu pada mereka. Dalam bahagian pertama kajian ini, infeksi sistemik untuk *C. parapsilosis* dan *C. tropicalis* telah dihasilkan di dalam tikus melalui cabaran intravena dan sifat patogenik mereka telah dinilai. Hasil kajian menunjukkan bahawa tikus yang dijangkiti dengan *C. parapsilosis* dan *C. tropicalis* memaparkan kadar jangka hayat hidup yang berbeza, dengan kematian hanya diperhati untuk tikus yang dijangkiti dengan *C. tropicalis*. Selain itu, tikus yang dijangkiti dengan *C. tropicalis* menunjukkan beban kulat yang lebih tinggi dan kerosakan ginjal yang lebih teruk. Secara amnya, hasil kajian ini menunjukkan bahawa *C. tropicalis* adalah lebih virulen daripada *C. parapsilosis* dan mencadangkan bahawa faktor virulen tertentu seperti morfogenesis menyumbang kepada perbezaaan patogenesis. Dalam konteks yang lain, kesukaran untuk mendiagnos candidiasis secara berkesan telah mengesa pencarian penanda biologi untuk penyakit ini. Squalene synthase adalah satu protein antigen yang ditemui daripada satu penyelidikan sebelum ini. Untuk menyiasat potensi protein ini sebagai penanda biologi, protein ini telah diekspresi di dalam *Pichia pastoris* dan protein rekombinan tersebut telah dipurifikasi dengan kromatografi afinitas. Keputusan ujikaji menunjukkan bahawa protein rekombinan yang dipurifikasi ini dikenal secara khas oleh antibodi poliklonal daripada tikus yang dijangkiti dengan *C. tropicalis* di atas Western blot, dengan itu mencadangkan bahawa protein ini berpotensi sebagai penanda biologi untuk *C. tropicalis*. Namun begitu, ujian lanjut masih diperlukan untuk mengesahkan kebolehgunaannya. Seterusnya, untuk menemui penanda biologi protein untuk *C. parapsilosis* dan *C. tropicalis* dan memahami interaksi antara hos dengan patogen dengan lebih lanjut, analisis immunoproteomik telah dijalankan. Untuk tujuan ini, pecahan sampel yang

diperkaya dengan protein dinding sel telah diimbas secara sistematik untuk mengesan antigen dengan menggunakan antiserum daripada tikus ujian. Analisis ini telah membawa kepada penemuan 12 protein immunogenik masing-masing untuk *C. parapsilosis* dan *C. tropicalis*, di mana 8 protein merupakan antigen yang sama untuk kedua-dua spesies ini. Antara antigen-antigen ini, 14 daripadanya telah dilaporkan sebelum ini sebagai antigen untuk *C. albicans*, manakala isocitrate dehydrogenase (Idh2p) dan dihydrolipoyllysine-residue succinyltransferase (Kgd2p) adalah pertama kali dilaporkan sebagai protein immunogenik untuk spesies *Candida*. Kerja kajian ini menunjukkan bahawa antigen-antigen ini telah diekspresi secara *in vivo* semasa infeksi dan berkemungkinan memainkan peranan yang penting dalam patogenesis. Seterusnya, antigen-antigen yang baru dilaporkan ini, Idh2p dan Kgd2p telah diekspresi di dalam *Escherichia coli* dan kemudian dipurifikasi dengan kromatografi afinitas. Sifat antigenik protein rekombinan ini telah disahkan dengan immunoblotting dengan menggunakan antiserum daripada tikus yang dijangkiti. Kajian awal ini mencadangkan bahawa protein-protein ini berpotensi untuk diaplikasikan sebagai penanda biologi untuk *C. parapsilosis* dan *C. tropicalis*. Namun begitu, kajian tambahan diperlukan untuk menilai kegunaan protein-protein ini. Secara kolektifnya, hasil penemuan daripada model infeksi tikus dan pemprofilan antigen dengan immunoproteomik membantu dalam meningkatkan pemahaman mengenai tindak balak hos terhadap jangkitan *C. parapsilosis* dan *C. tropicalis* serta membawa kepada penemuan antigen-antigen baharu untuk digunakan sebagai penanda biologi. Kerja kajian ini juga menerangkan penghasilan beberapa protein rekombinan antigenik yang menyediakan asas untuk penyelidikan lanjut.

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I certify that a Thesis Examination Committee has met on 5 June 2014 to conduct the final examination of Lee Pey Yee on her thesis entitled "Identification of Protein Biomarkers for *Candida parapsilosis* and *Candida tropicalis*" 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.

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LIST OF ABBREVIATIONS

2-DE	Two-dimensional gel electrophoresis
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSA	Bovine serum albumin
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CFU	Colony forming unit
CWP	Cell wall protein
Da	Dalton
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray ionization
H&E	Hematoxylin and eosin
HRP	Horseradish peroxidase
IEF	Isoelectric focusing
IgG	Immunoglobin G
IPG	Immobiline pH gradient
ITS	Internal transcribed spacer
LB	Luria-Bertani
MALDI	Matrix assisted laser desorption ionization
MS	Mass spectrometry
Mr	Molecular mass
NCBI	National Center for Biotechnology Information
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PAS	Periodic acid schiff
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pI	Isoelectric point
PMSF	Phenylmethylsulfonyl fluoride
PVDF	Polyvinylidene fluoride
SDA	Saboraoud dextrose agar
SDB	Saboraoud dextrose broth
SDS	Sodium dodecyl sulfate
Taq	*Thermus aquaticus*
TBP	Tributylphosphine
TBS	Tris-buffered saline
TCA	Trichloroacetic acid
TOF	Time-of-flight
YPD	Yeast Extract-Peptone-Dextrose

CHAPTER 1

INTRODUCTION

Candida species are commensal fungi in healthy individuals but are capable of causing opportunistic human infection and disseminating to deep tissues in susceptible populations. Hospitalized patients with immune deficiency or on prolonged antibiotics treatment or those receiving intravenous devices are particularly at risk for the potentially fatal systemic candidiasis (Chowta *et al*., 2007). To date, systemic candidiasis is the leading fungal bloodstream infection and its incidence has continued to increase over the past few decades (Falagas *et al*., 2010). Besides, the fact that systemic candidiasis is often associated with substantial morbidity and with attributable mortality of up to 45% also draw considerable concern (Eggimann *et al*., 2003). To worsen the situation, effective and sensitive diagnosis for systemic candidiasis is still lacking. Moreover, antifungal treatment has been frequently delayed due to difficult diagnosis and severe side effects have been reported following usage of the antifungal drugs (Pappas *et al*., 2009).

There are numerous efforts being carried out in the past to improve or complement diagnosis by blood culture method, which is the current gold standard for diagnosing systemic candidiasis. Regarding this, non-culture methods based on the detection of various fungal components have shown encouraging performances (Ahmad and Khan, 2012). Among these methods, detection assays based on antibody recognition against defined recombinant antigens have shown promising results in providing early diagnosis and even in identifying culture-negative cases (Clancy *et al*., 2008).

Biomarker is any molecules that may reflect a particular biological condition. As such, measurement of biomarkers can be exploited as diagnostic or predictor tool in clinical laboratories. Proteins are the final cellular products that carry out numerous biological functions as well as participate in the disease processes. Hence, identification of protein biomarkers has come to the forefront as a possible solution for current problems associated with delayed or non-specific diagnosis of candidiasis. The discovery of protein biomarkers is hoped to aid in detecting patients with infection for early initiation of antifungal therapy to achieve favorable clinical outcome. Nowadays, this endeavor is greatly facilitated by the availability of proteomic technology that offer powerful tool for global profiling of protein expression and identification of disease associated protein biomarkers. In fact, through proteomic analyses, several protein biomarkers have been identified for *Candida* and tested clinically. In a recent analysis, serum IgG antibody reactivity to Met6p, Hsp90p, Pgk1p, Ssb1p and Gap1p were found to be appealing as potential prognostic predictors for patients with systemic candidiasis (Pitarch *et al*., 2011).

It is fascinating that many *Candida* species are capable of switching from commensal organisms into harmful pathogens. To be a successful pathogen, *Candida* expresses numerous virulence factors that are tightly regulated throughout the course of

infection. It has been recognized that attachment of *Candida* to various host components is an important step to initiate infection, which is mediated by the expression of surface molecules known as adhesins (Sundstrom, 2002). As the infection progresses, *Candida* produces and releases hydrolytic enzymes such as secreted aspartyl proteinases to invade host tissues and contribute to the development of disseminated infection (Naglik *et al*., 2003). To persist inside the host, *Candida* adopts different strategies to overcome host immune attack (Jiménez-López and Lorenz, 2013). Besides, several lines of evidence also suggest that morphological transition from yeast to filamentous form is an important pathogenic trait (Lo *et al*., 1997; Phan *et al*., 2000; Kumamoto and Vinces, 2005). Nevertheless, our current understanding on virulence factors for *Candida* is still imperfect and is predominantly derived from studies on *Candida albicans*.

As the predominant *Candida* species, *Candida albicans* has become the major subject of study in different areas of research. Little attention has been paid to other *Candida* species and knowledge on their pathogenesis and protein biomarkers are still elusive. Furthermore, different *Candida* species are also known to differ considerably from each other in terms of their virulence attributes. On top of that, non-*albicans Candida* species especially *Candida parapsilosis* and *Candida tropicalis* are emerging recently as important pathogens in Malaysia and in several other countries that definitely deserve the research focus (Nucci and Colombo, 2007; Pfaller and Diekema, 2007; Rahman *et al*., 2008; Hamid *et al*., 2012). Thus, this project was conducted to shed light on *C. parapsilosis* and *C. tropicalis* as two increasingly prevalent pathogens that have not been widely studied before. The entire project encompasses several chapters and is detailed as below.

Mouse model of systemic candidiasis represents a valuable model that can recapitulate human infection. The first part of this study was carried out with the goal to investigate the pathogenicity of *C. parapsilosis* and *C. tropicalis* in a mouse model of systemic candidiasis. The pathological consequences following inoculation of the two *Candida* species were assessed and compared.

On the other hand, a previous study by our group has demonstrated that squalene synthase was a novel protein antigen that is involved in eliciting immune response in a mouse model of systemic *C. tropicalis* infection. Hence, the second part of this project was undertaken to express squalene synthase as recombinant protein in *Pichia pastoris* and to investigate its reactivity with immune sera from infected mice.

Exploration of *Candida* proteome is fundamental to understand the complex host-pathogen interaction at protein level in order to discover protein molecules that are important for pathogenesis. Besides, knowledge on protein antigens that participate in the disease process is useful to facilitate the identification of diagnostic markers and drug targets. So far, relatively little is known about the antigenic profiles and protein biomarkers of *C. parapsilosis* and *C. tropicalis* despite their growing importance. Thus, the third part of this work was performed with the aim of finding immunogenic proteins of *C. parapsilosis* and *C. tropicalis* as potential biomarkers by

using serological proteome analysis. Samples enriched with cell wall proteins from *C. parapsilosis* and *C. tropicalis* were resolved by two-dimensional electrophoresis followed by immunoblotting using antisera from infected mice to profile their antigenic components.

Subsequently, the last part of this study was carried out to further characterize the newly found antigenic proteins. The selected immunogenic proteins were cloned and expressed in *Escherichia coli* to explore their antigenicity.

The general objective of this study was to discover immunogenic proteins of *C. parapsilosis* and *C. tropicalis* as potential biomarker candidates.

The specific objectives of this study were:

1) to study the relative pathogenicity of *C. parapsilosis* and *C. tropicalis* in a mouse model of systemic candidiasis
2) to clone, express and purify squalene synthase in *Pichia pastoris* expression system and evaluate its serological reactivity
3) to screen and identify antigenic proteins of *C. parapsilosis* and *C. tropicalis* by using immunoproteomics
4) to generate recombinant proteins of selected antigens in *Escherichia coli* expression system and analyze their antigenicity

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