UNIVERSITI PUTRA MALAYSIA

IDENTIFICATION OF PROTEIN BIOMARKERS FOR CANDIDA PARAPSILOSIS AND CANDIDA TROPICALIS

LEE PEY YEE

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IDENTIFICATION OF PROTEIN BIOMARKERS
FOR CANDIDA PARAPSILOSIS AND CANDIDA TROPICALIS

By

LEE PEY YEE

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

June 2014
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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

IDENTIFICATION OF PROTEIN BIOMARKERS FOR CANDIDA PARAPSILOSIS AND CANDIDA TROPICALIS

By

LEE PEY YEE

June 2014

Chair: Associate Professor Chong Pei Pei, PhD
Faculty: Medicine and Health Sciences

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 dihydrolipooyllysine-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

PENGENDALI PASTIAN PENANDA BIOLOGI PROTEIN UNTUK CANDIDA PARAPSILOSIS DAN CANDIDA TROPICALIS

Oleh

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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  
\textit{Taq}  \textit{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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