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DEVELOPMENT OF LOOP-MEDIATED ISOTHERMAL AMPLIFICATION TECHNIQUE FOR DETECTION OF CANDIDA GLABRATA

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By

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DEVELOPMENT OF LOOP-MEDIATED ISOTHERMAL AMPLIFICATION TECHNIQUE FOR DETECTION OF *Candida glabrata*

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August 2020

Chairman : Associate Professor Leslie Than Thian Lung, PhD
Faculty : Medicine and Health Sciences

The increasing emergence of systemic fungal infections directly correlates with the growing population of immunocompromised groups. *Candida glabrata* is increasingly essential, due to rising isolation frequency and resistance development to antifungals. The diagnostic sensitivity of microbial culture ("gold standard") method could miss up to 50% of the invasive candidiasis (IC) patients. Molecular diagnosis, particularly conventional PCR, is promising in diagnosing many infections, but sometimes limited in sensitivity, cost of the thermal cycler, and lack of quantification ability. The need to develop a sensitive, specific, and non–machine-dependent method that operates at isothermal temperature is imperative for prompt management and excellent clinical outcome. The study, therefore, developed the loop-mediated isothermal amplification (LAMP) method integrated with lateral flow immunoassay (LFA) techniques for point of care testing (POCT) detection of *C. glabrata*. Internal transcribed spacer (ITS) ribosomal DNA of *C. glabrata* ATCC 2001 reference strain was cloned to form a recombinant plasmid (pUC19-ITS) as a standard for LAMP assay evaluation. Three pairs of LAMP primers (FIP/BIP, F3/B3 and LF/LB) were designed, optimised, and evaluated to determine LAMP assay's sensitivity and specificity. The LF/LB were labelled with digoxigenin and biotin respectively for LFA. The detection limit of LAMP using 10-fold serial dilutions of recombinant plasmid and blood spiking experiment was conducted. The LAMP assay was optimised and evaluated using LFA test strip. The expected size of the recombinant plasmid was confirmed by linearisation with KpnI enzyme. Amplicon size (1049 bp) confirmed using M13 primers. The LAMP detection limit demonstrates the high sensitivity of 2.25×10^{-0} copies/µL with 1000 – fold compared to conventional PCR that indicates 2.25×10^{3} copies/µL. The LAMP assay analysis of DNA from spiked blood indicates a range of detection limit between 10^{6}-10^{1} CFU/mL using gel electrophoresis analysis. The PCR range detection limit was 10^{6}-10^{5} CFU/mL. Thus, LAMP also shows 10^{4}–fold better performance. The LAMP specificity assay accurately detected *C. glabrata* with no cross-reactivity with other *Candida* or mould clinical isolates tested. The

developed LAMP assay offers a rapid, simple, sensitive, and specific molecular test for *C. glabrata* detection. It would be a useful tool speeding management of *C. glabrata* invasive candidiasis patients through POCT approach.

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

PEMBANGUNAN TEKNIK "LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP)" UNTUK PENGESANAN *Candida glabrata*

Oleh

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Peningkatan kemunculan jangkitan kulat sistemik secara langsungnya berhubung dengan peningkatan populasi golongan imunokompromi. *Candida glabrata* menjadi semakin penting disebabkan oleh peningkatan frekuensi pengasingan dan perkembangan rintangan terhadap antikulat. Kepekaan diagnostik bagi kaedah kultur mikrob boleh menyebabkan 50% pesakit kandidiasis invasive (IC) tidak dapat dikesan. Diagnosis molekul, terutamanya PCR konvensional berpotensi dalam diagnosis untuk kebanyakan jangkitan, tetapi kadang-kadang teknik ini terhad kepada isu sensitiviti, kos alat kitar terma dan kekurangan kemampuan dalam pengkuantitian. Keperluan untuk membangunkan kaedah baru yang mudah, pantas, sensitif, khusus, dan tidak bergantung kepada mesin yang beroperasi pada suhu isoterma adalah sangat penting untuk tindakan segera dan hasil klinikal yang baik. Oleh itu, kajian ini telah membangun dan mengesahkan "loop-mediated isothermal amplification" (LAMP) yang disepadukan dengan teknik "lateral flow immunoassay" (LFA) untuk diagnosis "point of care testing" (POCT) *C. glabrata*. "Internal transcribed spacer" (ITS) DNA ribosomal dari strain rujukan *C. glabrata* ATCC 2001 diklonkan untuk menghasilkan plasmid rekombinan (pUC19-ITS) sebagai standard untuk penilaian ujian LAMP. Tiga pasang primer LAMP (FIP/BIP, F3/B3 dan LF/LB) direka, dioptimumkan dan dinilai untuk menentukan kespesifikan dan kepekaan ujian LAMP. LF/LB dilabel dengan digoksigenin dan biotin untuk LFA. Had pengesanan LAMP ditentukan dengan menggunakan teknik pencairan bersiri 10 kali ganda plasmid rekombinan dan "blood spiking". Ujian LAMP dioptimumkan dan dinilai menggunakan jalur ujian LFA. Ukuran saiz plasmid rekombinan disahkan oleh pelinearan dengan enzim KpnI. Saiz amplicon (1049 bp) disahkan dengan menggunakan primer M13. Had pengesanan LAMP menunjukkan kepekaan tinggi (2.25×10^{-0} salinan/µL) dan 1000 kali ganda lebin sensitif berbanding dengan PCR konvensional yang menunjukkan 2.25×10^{3} salinan/µL. Analisis ujian LAMP menggunakan DNA dari darah menunjukkan julat had pengesanan antara 10^{6}-10^{1} CFU/mL selepas analisis elektroforesis gel. Had pengesanan julat PCR adalah 10^{6}-10^{5} CFU/mL. Oleh itu,

LAMP juga menunjukkan prestasi 10^4 kali lebih baik. Ujian spesifik LAMP dapat mengesan *C. glabrata* secara spesifiknya tanpa kereaktifan silang dengan *Candida* atau kulat klinikal yang lain. Ujian LAMP memberikan ujian molekul yang cepat, mudah, sensitif dan khusus untuk pengesanan *C. glabrata*. Ujian LAMP akan menjadi alat yang berpotensi untuk mempercepatkan pengurusan pesakit kandidiasis invasif *C. glabrata* melalui pendekatan POCT.

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

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LIST OF ABBREVIATIONS

A	Ampere
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
Abs	Absorbance
A_{600nm}	Optical density at wavelength 600 nanometer
bp	Base pair
β	Beta
BSA	Bovine serum albumin
C	Concentration
Ca^{2+}	Calcium ion
°C	Degree celsius
CBS 138	Centraalbureau voor Schimmelcultures 138
CDS	Coding sequences
CO_2	Carbon dioxide
$CaCl_2$	Calcium chloride
ddH_2O	Double distilled water
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulphoxide
E. coli	*Escherichia coli*
EDTA	Ethylene-diamine-tetraacetic acid
g	Gram
HCl	Hydrochloric acid
HDA	Helicase-dependent amplification

H_2SO_4	Sulphuric acid
H_2O	Water
h	Hours
IPTG	Isopropyl β-D-1-thiogalactopyranoside
JMPP	Jabatan Mikrobiologi dan parasitology perubatan
kb	Kilobase
kDA	kilodaltons
KOH	Potassium hydroxide
L	Litre
LB	Luria-Bertani
LAMP	Loop-mediated isothermal amplification
M	Molar
mA	Milliampere
mM	Millimolar
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NAT	Nucleic acid testing
nm	Nanometer
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pUC19	Plasmid of the University of Califonia 19
RNA	Ribonucleic acid
rpm	Revolution per minute
RT – qPCR	Quantitative real-time polymerase chain reaction
ROS	Reactive Oxygen species

SDS	Sodium dodecyl sulphate
SNP	Single nucleotide polymorphism
SOC	Super optimal broth with catabolite repression
TAT	Turn-around-time
UKMSC	Universiti Kebangsaan specialist centre
UMMC	University Malaya Medical centre
X-gal	5 – bromo – 4 – chloro – 3 – indolyl – β – D – galactopyranoside
α	Alpha
µg	Microgram
µL	Microliter
µm	Micrometer
µmoles	Micromoles
%	Percentage

CHAPTER 1

INTRODUCTION

1.1 Background of the study

The growing concern about the continuous increase in the advent of systemic infections due to fungal species constitutes a severe threat to an ever-growing population of immunocompromised individuals and other individuals at risk (Perlin et al., 2017). Invasive candidiasis is an essential healthcare-related fungal infection caused by many *Candida* species, including *Candida albicans*, and other few non-*albicans* species. However, the prevalence varies depending on the geographical locations (Pappas et al., 2018). Among the non-*albicans* candidiasis causative agents include; *C. glabrata; C. krusei, C. tropicalis; C. parapsilosis; C. lusitaniae; C. rugosa* and recently *C. auris* (Whaley et al., 2017; Berkow and Lockhart, 2018*;* Vatanshenassan et al., 2019). *Candida glabrata* a healthy human microbiota that colonizes epithelial surfaces of the gastrointestinal tract and genital tract. It becomes opportunistic when the immune system turns compromised. Thus, it causes both superficial and systemic infections (Gerwien et al., 2016). The predisposing conditions include; tuberculosis (TB), human immunodeficiency virus (HIV) disease, autoimmune disease (AD) and cancer diseases (CD) (Jahanshiri et al., 2018). Other immunosuppressive conditions include the widespread use of steroids and broad-spectrum antifungal drugs (Costa et al., 2016).

The distribution rate of *Candida* species changes over time with an increase in *C. glabrata* and *C. parapsilosis* and decrease in *C. albicans*. The distribution variation observed among different countries suggests this argument. For example, *C. albicans* is still the most notable species worldwide, but *C. glabrata* is progressively emerging in the United States, Canada, and Northern Europe (Ghazi et al., 2019). The proportional increase in *C. glabrata* infection occurred in the USA, where it accounts for one-third or more of all candidaemia isolates. The *Candida glabrata* followed closely by *C. parapsilosis* accounts for a 15% increase of all *Candida* isolates within the study period.

In contrast to *C. albicans,* the rate declines to <50% of all *Candida* infections detected (Lamoth et al., 2018). The overall candidaemia distribution rates of all species are dependent on the geographical location and patient population (Lamoth et al., 2018). In recent studies, there is a significant rise in the detection frequency of mucosal and systemic infections caused by *C. glabrata* (Kasper et al., 2015). Global data showed an increase in antifungal resistance trend among *C. glabrata* isolates. Generally, invasive candidiasis causes high mortality rates among the cases. The success of clinical outcome depends on the early diagnosis and prompt antifungal therapy (Perlin et al., 2017). The disturbing emergence of multi-drug resistant *Candida* species such as *C. auris* recently demands an improved understanding of their epidemiology, diagnosis and treatment (Das et al., 2018; Sarma & Upadhyay, 2017).

The internal transcribed spacer (ITS) of ribosomal DNA was used as the target DNA for molecular detection due to its resolution in differentiating closely related fungal species. The ITS region of ribosomal DNA (rDNA) serves as a consistent genetic marker for sequence analysis. The strategy helps for accurate and rapid molecular identification of fungal pathogens infecting humans. This research aimed to design a LAMP detection method integrated with lateral flow immunoassay to diagnose systemic infection caused by *C. glabrata*. The experimentally designed research achieved through optimization and testing of blood spiked with *C. glabrata* ATCC strain. The clinical validation of the assay achieved using positive invasive candidiasis samples.

The most prominent among the molecular techniques is PCR because of its promising relevance in diagnosing so many infectious agents. It has some drawbacks ranging from cost, expertise necessity, specificity issue and lack of ability to make point of-care-testing (POCT). A solution towards the problem lies in the development of LAMP integrated with lateral flow immunoassay (LFA) techniques. Its rapidity, simplicity, requires no sophisticated machine are leading advantages. It also operates at isothermal temperature and tendency to be incorporated into POCT for easy diagnosis.

1.2 Problem statement

The recent emergence of non-*albicans* species associated with hospital-acquired invasive candidiasis increased worldwide (Bongomin et al., 2017). The five-year study review conducted in Malaysia reported a significant increase in *C. glabrata* proportion from 1.1% to 21.6% (Haydar, 2018). *Candida glabrata* shows low susceptibility to azoles and sometimes indicates total resistance to fluconazole. *Candida* species show variable susceptibility to different antifungal drugs. Consequently, highlighting the importance of correct species identification and rapid antifungal MIC determination for resistance mechanisms screening among the species in the clinical settings (Santhanam et al., 2013). The conventional blood culture method is the gold standard for diagnosing bloodstream infections caused by *Candida* species. Although it is cost-effective and straightforward, it is time-consuming (takes up to 5 days to confirm the negative result). It has reduced sensitivity (< 50%) in patients who have received previous antimicrobial treatment. The blood sample's low volume contains a low number of microorganisms for blood culture analysis (Arvanitis et al., 2014; Dalla-Costa et al., 2017). Other diagnostic methods employed such as microscopy and histopathology could not meet physicians' need for urgency in deciding patient treatment and management. The molecular approaches for fungal detection rely on diagnostic markers such as ITS rRNA, D1/D2 and IGS1/IGS2.

The ITS has the higher resolution among them. The recent molecular detection approach for fungal DNA is by real-time PCR. The report is superb in diagnosing bloodstream infections (BSI) because of its inherent rapidity, sensitivity, and specificity for identifying various infectious diseases (Van den Brand et al., 2014). However, it also suffers some limitations, i.e. cost, facility, and expertise (Van den

Brand et al., 2014; Dalla-Costa et al., 2017). Other nucleic acid amplifications become valuable tools in virtually all life science fields. It includes application-oriented fields such as clinical medicine to diagnose infectious diseases, genetic disorders, and genetic traits. Thus, the proposed study aimed to develop a rapid, simple, less costly molecular detection method (LAMP) to diagnose *C. glabrata* from invasive candidiasis patients.

1.3 Significance of the study

The research would positively impact the rapid diagnosis of *C. glabrata* from blood samples of invasive candidiasis patients. The research would help to increase speed, simplicity and sensitivity for prompt clinical decision and therapy initiation.

1.4 Objectives

1.4.1 General objective

The study aims to develop a LAMP molecular diagnostic technique which is simple, rapid, and sensitive for detection of *C. glabrata*. The method has reduced turn-around-time (TAT), less costly and allows for LFA point-of-care integration.

1.4.2 Specific objectives

1. To optimize the LAMP system for detecting *C. glabrata* using newly designed primer set targeting internal transcribed spacer (ITS2) of the ribosomal DNA.
2. To determine the analytical sensitivity and specificity of the LAMP method for detection of *C. glabrata*
3. To measure the detection limit of the established LAMP system on spiked blood samples.
4. To determine the LAMP assay's efficacy to detect *C. glabrata* from clinical blood samples of patients with invasive candidiasis.
5. To determine the LFA strip detection limit after LAMP assay for POCT detection of *C. glabrata*.

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