MOLECULAR IDENTIFICATION, ANTIFUNGAL SUSCEPTIBILITY AND VIRULENCE FACTORS OF *Candida glabrata* ISOLATED FROM MALAYSIAN PATIENTS

AZADEH LOTFALIKHANI

DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF MEDICAL SCIENCE

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Molecular identification, antifungal susceptibility and virulence factors of *Candida glabrata* isolated from Malaysian Patients

Field of Study: **Medical Microbiology**

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ABSTRACT

There has been an increase in the incidence of candidiasis among hospitalized patients in many healthcare institutions in recent years. Among the *Candida* species, *Candida glabrata* has been described as the second or third most common yeast species isolated from patients with vaginitis and invasive candidiasis. Accurate identification of *Candida* spp. from clinical specimens and antifungal susceptibility testing are important to facilitate optimal antifungal therapy and patient management. Molecular techniques are excellent tools for identification of new species and strain typing of yeasts. *In vitro* assessment of the virulence properties of a pathogen, for example, the ability of an organism to produce hydrolytic enzymes and biofilm, may provide some insights on the pathogenesis of the infection it causes. This study aims to determine the identification and the characteristics of *C. glabrata* isolated from various clinical specimens in the Diagnostic Microbiology Laboratory, University of Malaya Medical Centre, from 2008-2012. A total of 185 *C. glabrata* isolates were identified in this study based on sugar assimilation tests. Majority of the isolates were obtained from vaginal swabs (n=142, 76.8 %), followed by blood samples (n=11, 5.9%), urine (n=14, 7.6%), respiratory secretions (n=4, 2.2%), and others (n=14, 7.6%). The yeast DNA was extracted from each isolate and subjected to testing using a singleplex PCR assay for discrimination between *C. glabrata*, *C. bracarensis* and *C. nivariensis*, as reported by Enache-Angoulvant *et al.* (2011). Four isolates obtained from a blood specimen and three vaginal swabs of two patients were identified as *C. nivariensis*, a rare and emerging yeast pathogen. The identity of the isolates was confirmed based on sequence analysis of the yeast internal transcribed spacer (ITS) region and D1D2 domain. The low prevalence (1.1 %) of *C. nivariensis* isolates is in agreement with several studies reported in other geographical regions. Based on the ITS sequence data (ranging from 619 to 624 bp) obtained from 35 randomly selected *C. glabrata* isolates, a total of 9

sequence types were identified. All *C. glabrata* and *C. nivariensis* isolates were susceptible to amphotericin B, caspofungin, fluconazole, and voriconazole. Randomly amplified polymorphic DNA (RAPD) analysis of *C. glabrata* isolates using two single primers [M13 and $(GTG)_5$] identified six and five genetic clusters, respectively. *C. nivariensis* isolates formed a unique genetic cluster as compared to *C. glabrata* isolates. A total of 15 RAPD types of *C. glabrata* were identified in this study. Weak phospholipase activity and biofilm production was demonstrated by *C. glabrata* isolates investigated in this study. This study reports for the first time the identification of *C. nivariensis* in a Malaysian hospital. The yeast was misidentified as *C. glabrata* as it shared many phenotypic characteristics with *C. glabrata*. Despite of the genetic diversity of *C. glabrata* (as exhibited by sequence determination of the ITS region and RAPD analysis), all isolates are susceptible to the antifungal drugs tested in this study. Although *C. glabrata* is probably not as virulent as compared to *C. albicans* due to its low production of phospholipase and biofilm production, however; further work is necessary to validate the finding.

ABSTRAK

Terdapat peningkatan dalam kejadian kandidiasis di kalangan pesakit yang dimasukkan ke hospital dalam banyak institusi perubatan dalam tahun-tahun kebelakangan ini. Antara spesies *Candida*, *Candida glabrata* telah digambarkan sebagai spesies yis yang kedua atau ketiga kerap yang diasingkan daripada pesakit dengan vaginitis dan kandidiasis invasif. Pengenalpastian *Candida* spp. yang tepat daripada spesimen klinikal dan ujian kerentanan antikulat adalah penting untuk memudahkan terapi antikulat yang optimum dan pengurusan pesakit. Teknik molecular adalah aplikasi yang sangat baik untuk mengenalpasti spesies baru dan pengetipan strain yis. Penilaian ciri-ciri kevirulenan patogen secara *in vitro*, contohnya, keupayaan organisma untuk menghasilkan enzim hidrolisis dan biofilem, boleh memberi maklumat mengenai patogenesis jangkitan yang disebabkannya. Kajian ini bertujuan untuk menentukan pengenalpastian dan ciri-ciri *C. glabrata* yang diasingkan daripada pelbagai spesimen klinikal di Makmal Diagnostik Mikrobiologi, Pusat Perubatan Universiti Malaya pada 2008-2012. Seramai 185 *C. glabrata* telah dikenalpasti dalam kajian ini berdasarkan ujian asimilasi gula. Kebanyakan isolat telah diperolehi daripada swab vagina (n = 142, 76.8%), diikuti oleh sampel darah (n = 11, 5.9%), air kencing (n = 14, 7.6%), rembesan pernafasan (n = 4, 2.2%), dan lain-lain (n = 14, 7.6%). DNA yis telah diekstrak daripada setiap isolat dan ujian singleplex PCR dilakukan untuk mendiskriminasikan *C. glabrata, C. bracarensis* dan *C. nivariensis*, seperti yang dilaporkan oleh Enache-Angoulvant et al. (2011). Empat isolat yang diperolehi daripada spesimen darah dan swab vagina dua pesakit telah dikenalpasti sebagai *C. nivariensis*, sejenis patogen yis yang jarang dan baru ditemui. Identiti isolat dikenalpastikan berdasarkan analisis "internal transcribed spacer (ITS) region" dan domain D1D2. Kadar prevalens *C. nivariensis* yang rendah (1.1%) berbanding *C. glabrata* bersetuju dengan beberapa kajian yang dilaporkan di kawasan geografi yang lain. Berdasarkan data jujukan ITS

(antara 619-622 bp) yang diperolehi dari 35 isolat yang dipilih secara rawak, sebanyak 9 jenis "sequence type" telah dikenal pasti. Kerentanan isolat *C. glabrata* dan *C. nivariensis* terhadap amphotericin, caspofungin, fluconazole dan voriconazole ditentukan dalam kajian ini. Analisis "randomly amplified polymorphic DNA (RAPD)" dilakukan dengan primer [M13 dan $(GTG)_5$] mengenalpasti enam dan lima kluster genetik. Sejumlah 15 RAPD type telah dikenalpasti di kalangan *C. glabrata*. Isolat *C. nivariensis* membentuk kelompok genetik yang unik berbanding *C. glabrata*. Pengeluaran aktiviti fosfolipase dan biofilem yang lemah telah dipamerkan dalam *C. glabrata* dalam kajian ini. Kajian ini melaporkan buat pertama kali mengenai pengenalpastian *C. nivariensis* di sebuah hospital di Malaysia. Yis ini telah disalah anggap sebagai *C. glabrata* kerana ia berkongsi banyak ciri-ciri fenotip dengan *C. glabrata*. Walaupun kepelbagaian genetik *C. glabrata* (seperti yang dipamerkan oleh penentuan urutan "ITS region" dan analisis RAPD), semua isolat suseptibel kepada ubat-ubatan antikulat yang diuji dalam kajian ini. Walaupun *C. glabrata* mungkin kurang virulen berbanding *C. albicans* disebabkan oleh pengeluaran fosfolipase dan biofilem yang rendah, kajian lanjut diperlukan untuk mengesahkan penemuan ini.

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LIST OF SYMBOLS AND ABBREVIATIONS

_	Negative
%	Percent
5-FC	5-Flucytosine
(-COOH)	carboxylic acid group
=O	ketone group
<	less than
>	more than
≥	equal or more than
C^{o}	Celsius
α	alpha
β	beta
µg	microgram
µg/ml	microgram per milliliter
µl	microliter
µM	micromolar
µm	micrometer
AIDS	Acquired Immunodeficiency Syndrome
AM	Amphotericin B
B/C	Blood Culture
BLAST	Basic Local Alignment Search Tool
bp	base pair
BSI	Bloodstream infection
C. albicans	*Candida albicans*
C. dubliniensis	*Candida dubliniensis*
C. glabrata	*Candida glabrata*
C. guilliermondii	*Candida guilliermondii*
C. krusei	*Candida krusei*
C.parapsilosis	*Candida parapsilosis*
C. bracarensis	*Candida bracarensis*
C. nivariensis	*Candida nivariensis*
C. norvegensis	*Candida norvegensis*
Cas	Caspofungin
CFUs	colony forming units
CLSI	Clinical and Laboratory Standards Institute
CSH	Cell surface hydrophobicity
Clo	Clotrimazole
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTPs	Deoxyribonucleoside triphosphate
EK	electrophoretic karyotyping
EDTA	Ethylenediaminetetracetic acid
EDX	energy-dispersive X-ray spectroscopy
et al	et alia (and others)
FL	Fluconazole
g	gram
h	hour
H_2O	water
HIV	human immunodeficiency virus
HVS	High Vaginal Swab
ICU	Intensive Care Unit
i.e.	id est (that is)
ITR	itraconazole

ITS	internal transcribed spacer
L	liter
M	Molar
LVS	Low Vaginal Swab
mg/l	milligram per liter
mg/ml	milligram per milliliter
$MgCl_2$	magnesium chloride
MIC	minimum inhibitory concentration
min	minute
ml	milliliter
mM	millimolar
MOPS	3-(N-Morpholino) propanesulfonic acid 4-Morpholinepropanesulfonic acid
MRL	Mycology Reference Laboratory
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-Diphenyltetrazolium bromide
NaCl	sodium chloride
NCAC	non-*Candida albicans Candida* spp.
NCBI	National Institute of Standards and Technology
nm	nanometer
No.	Number
OD	Optical density
OPC	oropharyngeal candidiasis
PBS	Phosphate-buffered saline
PCR	polymerase chain reaction
PDA	potato dextrose agar
R	resistant
RFLP	restriction fragment length polymorphisms
RNA	ribonucleic acid
rpm	revolution per minute
RPMI	Roswell Park Memorial Institute medium
RAPD	Random Amplified Polymorphic DNA
rDNA	ribosomal DNA
rRNA	Ribosomal RNA
s	second
S	Susceptible
S-DD	Susceptible Dose-Dependent
SDA	Sabouraud's dextrose agar
SEM	Scanning Electron Microscopy
sp.	Species
TM	melting temperature
TBE	Tris-borate-EDTA
UMMC	University Malaya Medical Centre
USA	United States of America
USDA	US Department of Agriculture
V	voltage
v/v	volume per volume
VVC	vulvovaginal candidiasis
VO	Voriconazole
w/v	weight per volume
XTT	2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H- Tetrazolium-5-carboxanilide
YEPD	yeast-extract peptone dextrose agar

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CHAPTER 1

INTRODUCTION

1.1 General Introduction

Candida spp. is a commensal organism which is found in 80% of human population (mainly in the mouth, vagina and gastrointestinal tracts) (Zadik *et al.*, 2010). It is a yeast-like organism, typically measures 3–4 µm in diameter. The infections caused by *Candida* species are in general referred as candidiasis. The clinical spectrum of candidiasis is extremely diverse. Almost any part of organ or system in body can be infected (Guarner & Brandt, 2011). Candidiasis may be superficial or disseminated. Disseminated infections arise from haematogenous spread from the primarily infected locus (Nucci *et al*., 2003). Candidiasis is mostly an endogenous infection, which frequently occurs due to the overgrowth of the organisms inhabiting as normal flora. However, it may occasionally be acquired from exogenous sources or by host contact (Walsh *et al*., 2004).

C. albicans is the most pathogenic and most commonly encountered species among all of the *Candida* species (Walsh *et al.*, 2004). However, other species such as *C. glabrata*, *C. krusei*, and *C. tropicalis* are also frequently reported (Kent, 1991; Sim & Hughes, 1998; J. D. Sobel, 1997). Previously, *C. glabrata* has been ranked fourth among *Candida* species (third in patients who have undergone surgery) and is associated with an equally high mortality rate with *C. albicans* (Fraser *et al.*, 1992; Komshian *et al*., 1989; Wingard *et al*., 1993; 1994). However, recent investigation suggests that it is the second most important etiologic agent of invasive candidiasis (Fraser *et al*., 2012; Malani *et al.*, 2005; Pfaller & Diekema, 2007). Additionally, *C. glabrata* is of special importance because of its innately increased resistance to

antifungal agents, specifically the azoles (Pfaller & Diekema, 2007; Takahashi *et al.,* 2012).

C. glabrata has been classified in the '*glabrata* group' of the genus *Nakaseomyces* (Kurtzman, 2003) which also includes *Candida nivariensis* and *Candida bracarensis* (Alcoba-Florez *et al*., 2005; Correia *et al.,* 2006; Sharma *et al.,* 2013). Both *C. nivariensis* and *C. bracarensis* have been reported as emerging pathogenic yeasts that are phenotypically indistinguishable from *C. glabrata* (Alcoba-Florez *et al.,* 2005; Correia *et al.,* 2006). The clinical importance of *C. nivariensis* has been recognized following isolation of the yeast from blood cultures and deep sterile body sites of patients and resistance to antifungal drugs (Borman *et al.,* 2008; Fujita *et al.,* 2007; Lockhart *et al.,* 2009). *C. bracarensis* has been described for the first time from vagina and blood culture of patients in Portugal and United Kingdom (Correia *et al.,* 2006).

Definite identification of *C. glabrata, C. nivariensis* and *C. bracarensis* can only be performed using molecular techniques. Recently, a singleplex PCR assay using primers targeting a protein component of the 60S ribosomal subunit (*RPL31*) gene has been validated for discrimination of these closely related species (Enache-Angoulvant *et al.,* 2011). Several molecular methods have been used to distinguish the genetic difference amongst *Candida* species. PCR fingerprinting is a major typing technique which is rapid, sensitive and reliable in distinguishing genetic differences and thus, is able to provide important epidemiological data on the distribution of *Candida spp.*

The high incidence of *C. glabrata* could be attributed to the ability of the organism to form biofilms and resistant to traditional antifungal therapies, especially in patients with immunosuppression (Rodrigues *et al*., 2014). The virulence of *Candida* species is not well defined. To date, the production of hydrolytic enzymes such as secretory aspartyl proteinases, phospholipase enzymes and adherence to host tissues

have been regarded as the major determinants of the pathogenicity of *C. albicans* (Ghannoum,2000; LaFleur *et al.*, 2006; Naglik *et al.*, 2004; Schaller *et al.*, 2005). However, little is known on the pathogenicity of other *Candida* species, including *C. glabrata.* Additionally, drug resistance has been identified as the major cause of treatment failure among patients with candidiasis. Candidiasis is usually treated using amphotericin B, fluconazole and itraconazole (Faria *et al*., 2011). However, resistance to fluconazole and voriconazole and decreased susceptibility to posaconazole, has been noted among *C. glabrata* isolates in China (Pfaller *et al*., 2015). The emergence of resistance to amphotericin B and azole drugs has also been reported in *C. bracarensis* (Warren *et al.,* 2010). Antifungal susceptibilities of *Candida* species can be determined using E-test method or microbroth dilution method.

1.2 Justification

There is little data about the type and distribution of *C. glabrata* among patients with candidiasis in Malaysia. A study conducted 15 years ago reported that 11.7% of *Candida* isolates from vaginal swabs of patients was *C. glabrat*a (Ng *et al.*, 2001). In another study, Chong *et al*. (2003) reported that 14.9% of recurrent vulvovaginal candidiasis (VVC) were caused by *C. glabrata*. It has been reported that *C. glabrata* infections are difficult to treat due to the reduced susceptibility of the organism to many azole antifungal agents, especially fluconazole (Hitchcock *et al*., 1993; Komshian *et al*.,1989; Willocks *et al*., 1991). In Malaysia, *C. glabrata* has been reported to be the most resistant *Candida* species against itraconazole, posaconazole, fluconazole and ketoconazole (Santhanam *et al*., 2013). Very few studies described the genetic diversity and virulence properties of *C. glabrata*. There is no information on the occurrence and antifungal susceptibility profiles of *C. nivariensis* and *C. bracarensis* in our clinical setting.

The antifungal susceptibilities, genetic heterogeneity and the study on the virulence of *C. glabrata* will help our understanding on the epidemiology and pathogenicity of the infection.

1.3 Objectives:

The objectives of this study are:

i. to identify *C. glabrata* using phenotypic and genotypic approaches

ii. to determine genetic relatedness of *C. glabrata* isolated from different anatomic sites

iii. to determine antifungal susceptibilities of *C. glabrata*

iv. to determine possible virulence factors of *C. glabrata*

CHAPTER 2

LITERATURE REVIEW

2.1 Candidiasis

Candida is a yeast-like, saprophytic fungus which sometimes causes thrush, or invasive infection (Walsh *et al*., 1996). The disease it causes, candidiasis, is also known as candidosis, moniliasis, and oidiomycosis (James et *al*., 2015). Candidiasis is classified into either mucosal or systemic infections. Mucosal infections generally affect gastrointestinal epithelial cells, vaginal, or oropharyngeal mucosa (Kabir *et al*., 2012). Thrush which is the best known mucosal infections is characterized by white spots on the infected membranes. Meanwhile, systemic *Candida* infection is common to immunocompromised individuals such as human immunodeficiency virus (HIV)-infected patients, transplant recipients, chemotherapy patients, and low-birth weight neonates (Pfaller *et al.*, 2006; Schelenz *et al*., 2009).

Among the members in the genus *Candida*, *C. albicans* is a normal constituent of the human flora. Other clinically important *Candida* spp. include *C. glabrata, C. rugosa, C. parapsilosis, C. tropicalis, C. krusei, C. dubliniensis, C. guilliermondii* and *C. kefyr* (D'Enfert *et al*., 2009; Pfaller *et al*., 2006). *C. albicans* is the most pathogenic and most commonly encountered species among all of these species (Walsh *et al*., 2004).*C. glabrata* has recently gained recognition as the second most important etiologic agent of invasive candidiasis (Fraser *et al*., 2012; Malani *et al*., 2005; Pfaller & Diekema, 2007). Recently, the prevalence of *C. glabrata* infections has been reported to range from 24.3% to 26 % in North American hospital setting (Pfaller *et al*., 2004; Trofa *et al*., 2008; Wilson *et al*., 2014) and it is currently ranked as the second or third most important causative agent of superficial (oral, esophageal, vaginal, or urinary) or systemic candidal infections (Wilson *et al*., 2014). The widespread immunosuppressive agents therapy has caused an increase the frequency of vaginal, oral and urinary tract infection caused by this *Candida* species (Fidel *et al*., 1999; Li *et al*., 2007) postulated

that the majority of the population is asymptomatically colonized by both *C. albicans* and *C. glabrata.* Under certain predisposing factors, such as treatment with antibiotics, diabetes, cancer, extreme age, immunosuppression, intravenous catheters or long-term hospitalization, *C. glabrata* can cause superficial or life-threatening systemic infections, contributing to high rates of morbidity and mortality amongst hospitalized patients (Kourkoumpetis *et al*., 2011; Li *et al*., 2007b; Perlroth *et al*., 2007).

2.2 Taxonomy aspect of *Candida glabrata*

Historically, *C. glabrata* was classified in the genus *Torulopsis* due to its inability in pseudohypha production (Zwillenberg, 1966). However, it was later determined that the ability to produce pseudohyphae was not reliable to distinguish members of the genus *Candida*, and thus, *T. glabrata* was reclassified in the genus *Candida* (Odds, 1988). Together with three sister species, *Kluyveromyces delphensis, Candida castellii* and *Kluyveromyces bacillisporus*, *C. glabrata* has been classified under the genus *Nakaseomyces* (Kurtzman, 2003). *C. nivariensis* and *Candida bracarensis* have later been added to the same genus (Alcoba-Florez *et al*., 2005; Correia *et al.,* 2006; Sharma *et al.,* 2013). The genus *Nakaseomyces* has been further differentiated into two subgroups, with the 'glabrata group' being the first group to include the three pathogenic species, *N. delphensis* and *C. castellii*, and the second group, which include *N. bacillisporus* (Ahmad *et al*., 2014). The genome of *C. glabrata* (CBS138/American Type Culture Collection, ATCC2001) has been sequenced. It has 13 chromosomes with the size ranging from 0.5-1.4 Mb (Dujon *et al*., 2004).*C. glabrata* are phylogenetically, genetically and phenotypically very different from *C. albicans* (Brunke & Hube, 2013).

Firstly, *C. glabrata* is a haploid organism, whereas C. *albicans* is a diploid, polymorphic fungus. *C. albicans* switches from yeast to hyphal (and pseudohyphal) forms, whereas *C. glabrata* is a strictly haploid organism and grows only in the yeast form (Kaur *et al*., 2005).

Taxonomically, *C. albicans, C. parapsilosis* and *C. tropicalis* belong to the relatively closely related members of a group called the CUG clade (sharing a unique codon exchange from leucine to serine), while *C. glabrata* is a 'misnomer' which is much more closely related to the baker's yeast *Saccharomyces cerevisiae* than to *C. albicans* (Dujon *et al*., 2004). *C. glabrata* is also distinguishable from *C. albicans* by its small-subunit 18S rRNA after cellular rRNA isolation and sequencing (Barns *et al*., 1991). Figure 2.1 shows the phylogenetic positions of *C. glabrata* and other Candida species based on genome sequence analysis (A. BIALKOVÁ, 2006).

C. glabrata shares a common ancestor with *Saccharomyces cerevisiae* but has lost many genes compare to *S. cerevisiae,* and hence, smaller than those of *S. cerevisiae* (Dujon *et al*., 2004). As a result, the genome has been decreased to a haploid form leading to the complete loss of some metabolic pathways. The lost genes include those needed for the metabolism of galactose (*GAL1, GAL7, GAL10*), phosphate (*PHO3, PHO5, PHO11, PHO12*), nitrogen (*DAL1, DAL2*), sulfur metabolism (*SAM4*), and pyridoxine biosynthesis (*SNO1, SNO2, SNO3*) (Kaur *et al.*, 2005; Butler *et al.*, 2009). It has been postulated that the reduction in the genome size may have changed *C. glabrata* to a pathogenic yeast (Dujon *et al.*, 2004; Fidel *et al.*, 1999).

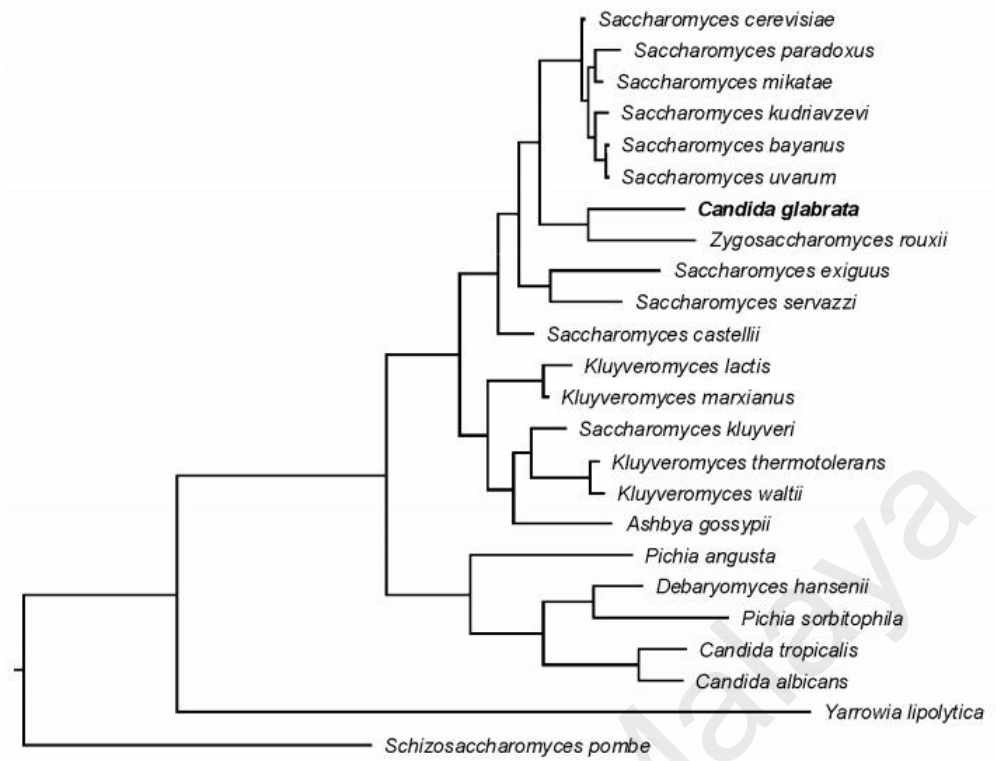


Figure 2.1: Phylogenetic tree of well-known yeast species (A. BIALKOVÁ, 2006)

2.3 *Candida nivariensis* and *Candida bracarensis*

Based on recent genetic studies, two closely related members of *C. glabrata, i.e., C. nivariensis* and *C. bracarensis* have been reported as emerging pathogenic yeasts that are phenotypically indistinguishable from *C. glabrata* (Alcoba-Florez *et al*., 2005; Correia *et al*., 2006). The clinical importance of *C. nivariensis* has been recognized following isolation of the yeast from blood cultures and deep sterile body sites of patients and resistance to antifungal drugs (Borman *et al*., 2008; Fujita *et al*., 2007; Lockhart *et al*., 2009). *C. bracarensis* has been described for the first time from the vagina and blood culture of patients in Portugal and United Kingdom (Correia *et al*., 2006). *C. nivariensis* and *C. bracarensis* are presumptively identified based on the production of white colonies on CHROMagar medium, microscopic features of small budding yeast cells and a positive rapid trehalose test (Alcoba-Florez *et al*., 2005; Bishop *et al*., 2008; Correia *et al*., 2006; Warren *et al*., 2010). In laboratories where conventional routine identification such as chromogenic media, rapid trehalose

assimilation test, biochemical panels are used, *C. nivariensis* and *C. bracarensis* strains can be misidentified as *C. glabrata* (Enache-Angoulvant *et al.*, 2011). Hence, molecular techniques are used for definite identification of *C. glabrata, C. nivariensis* and *C. bracarensis*. Recently, a single-plex PCR assay using primers targeting a protein component of the 60S ribosomal subunit (RPL31) gene has been validated for discrimination of these closely related species (Enache-Angoulvant *et al*., 2011).

Due to the pathogenic properties of *C. nivariensis* and *C. bracarensis*, researchers have started looking for the two newly emerging yeasts by re-examining *C. glabrata* isolates, especially in patient with low immune defence system and severe haematological malignancies (Warren *et al.*, 2010).

2.3.1 *C. nivariensis*

C. nivariensis is an opportunistic fungus initially isolated from three patients in Spanish hospital in 2005. As the yeast was also reported from flowers in Canada, it was thus suggested these patients might be infected from hospital garden or potted plants (Borman *et al.*, 2008; Lachance *et al.*, 2001). In 2006, 16 *C. nivariensis* isolates cultured from deep and sterile sites of the body were identified at the Mycology Reference Laboratory (MRL) in United Kingdom. These isolates were identified as *C. nivariensis* by sequencing of the internal transcribed spacer 2 (ITS2) regions (Borman *et al.*, 2008). Recently, *C. nivariensis* have been identified in 4 Indian patients with vulvovaginal candidiasis (Sharma *et al*., 2013).

2.3.2 *C. bracarensis*

C. bracarensis is another pathogenic fungus related to *C. glabrata* genetically. This new invasive fungus was described in 2006 following its isolation from a patient in Portuguese hospital who was suffering from vaginal candidiasis. As the fingerprinting pattern and 26S rDNA D1/D2 region of the isolate was different and quite unique, the yeast was proposed as a novel species (Correia *et al.*, 2006). Subsequently, seven

isolates have been described in the literature, with the isolates originated from blood, throat, stool and vaginal cultures (Lockhart *et al.*, 2009).

2.4 Infections caused by *C. glabrata*

i) Vulvovaginal candidiasis

Most (80%–85%) cases of vulvovaginal candidiasis (VVC) are caused by *C. albicans* (Fraser *et al*. 1992; Malani *et al*., 2005; Pfaller *et al*., 2006). Most of the infections with non-albicans species of *Candida* are due to *C. glabrata* (5%–10% of cases) (Singh *et al*., 2002). The increased use of short courses of both topical and oral azole antimycotic regimens is thought to be the reason for the increased incidence of vaginitis caused by non-albicans *Candida* spp.

C. glabrata is the most common non-albicans *Candida* spp. (Cauwenbergh & Heykants, 1990; Sobel 1997), isolated particularly from women receiving long-term maintenance low-dose fluconazole prophylactic regimens (Lynch *et al*., 1996). Vermitsky *et al.* (2008) reported that *C. glabrata* contributed to 7.9% of positive vaginal swabs in a US study. The study also observed an increase prevalence of *C. glabrata* in aged population group (more than 20%) compared to that of *C. albicans*. In China, the prevalences of *C. glabrata* were higher amongst patients with VVC (Liu *et al*., 2014; Zhang *et al*., 2014). An Indian study shows the predominance of *C. glabrata* species (56.25 %), followed by *C. tropicalis* and *C. krusei* (Gupta *et al*., 2015). Additionally, diabetic patients with VVC were predominantly infected by *C. glabrata*; a total of 61.3% of diabetic patients have been reported to contract the infection in India (Goswani *et al*., 2006; Ray *et a*l., 2007). A total of 34.5% of VVC infections in Turkey were caused by *C. glabrata* (Gultekin *et al*., 2005). A Malaysian study conducted 15 years ago reported that 11.7% of *Candida* isolates from vaginal swabs of patients was

C. glabrata. In another study, Chong *et al*. (2003) reported that 14.9% of recurrent VVC were caused by *C. glabrata*.

A variety of risk factors have been associated with *C. glabrata* vaginitis, including older patients, underlying medical conditions such as uncontrolled diabetes mellitus, and douching (Geiger *et al*., 1995). Vaginal pH in *C. glabrata* infection case has been reported to be similar to the vaginal pH in untreated *Candida* vaginitis characterized by vaginal discharge which is vaginal normal pH (<4.5) (Ray *et al*., 2007). However, abnormal discharge and inflammation was less frequently reported in women with symptomatic vaginitis due to *C. glabrata*, in comparison to *C. albicans* (Geiger *et al*., 1995). This has been associated with the failure of the *C. glabrata* to form pseudohyphae and hyphae *in vivo*. Additionally, *C. glabrata* vaginitis is frequently reported with a burning sensation rather than itch. Interestingly the higher mycological cure (72.4%) to boric acid therapy have been observed in diabetic women with *C. glabrata* VVC (Ray *et al*., 2007).

ii) Oropharyngeal and esophageal infections

Oropharyngeal, esophageal candidiasis is other common mucosal infections caused by *Candida* spp. (Diekema *et al*., 2012). Dos Santos Abrantes *et al*. (2014) reported about 19.05% of mucosal infections in immunosuppressed patients in South Africa were associated with *C. glabrata*. The incidence of *C. glabrata* in Indian acquired immunodeficiency syndrome (AIDS) patients who had oropharyngeal candidiasis (OPC) was 7.5% (Baradkar & Kumar, 2009). Mixed infection of *C. glabrata* with *C. albicans* in oral cavity has been reported (Redding *et al*., 2002). In one study, *C. glabrata* strain recovered from esophageal surfaces and OPC is generally co-isolated with *C. albicans* (70 %) followed *by C. parapsilosis* (15%), and *C. tropicalis* (5%), respectively (Baradkar & Kumar, 2009).

iii) Systemic infections

C. glabrata candidemia can present as a low-grade fever to fulminant septic shock (Fidel *et al*., 1999). As there are no characteristic signs and symptoms in disseminated candidiasis, persistent fever can be the only manifestation. Few studies have evaluated the specific risk factors for *C. glabrata* candidemia. It is believed that the risk factors associated with the infection are similar to those caused by *C. albicans* infections, for instance, prolonged hospitalization and prior antimicrobial use (Vazquez *et al*., 1998). As *C. glabrata* is known to demonstrate reduced susceptibility to fluconazole, this can result in treatment failures when employing the antifungal agent for empirical treatment of *Candida* bloodstream infection (Foster *et al*., 2007).

Perlroth *et al*. (2007) reported that *C. glabrata* is the second most common isolates in systemic candidiasis in the USA. Together with C. *albicans, C. glabrata* accounted for approximately 65%–75% of all cases. In India, it was the third most common *Candida* species causing candidemia in an Indian intensive care unit (ICU) with an incidence of 0.21% of 1000 ICU admissions and 30 days of mortality rate (Gupta *et al*., 2015). In another investigation, *C. glabrata* is responsible for approximately 26% of *Candida* bloodstream infections in the United States and accounted for up to 50% of mortality rates, higher than those associated with *C. albicans* (Horn *et al*., 2009). As high as 64% of mortality rate due to *C. glabrata* fungemia has been reported in USA (Malani *et al*., 2005).

iv) Urinary tract infection

Approximately 50% to 70% of urinary isolates of *Candid*a are due to non-albicans *Candida* species, of which the most common isolate is *C. glabrata* (Achkar & Fries, 2010). In a large multicenter study, *C. glabrata* was responsible for 20% of the *Candida* urinary tract infections (Sobel *et al*., 2000). *C. glabrata* is usually part of a polymicrobial infection, including other bacterial uropathogens or a second *Candida* species (usually *C. albicans*). Similar to *C. albicans* urinary tract

infections, the majority of *C. glabrata* urinary tract infections occur in elderly hospitalized, debilitated, and catheterized patients who have recently received antibacterial agents (Fidel *et al.*, 1999).

v) Other infections

From 1978 to 2010, about 12 cases of endophthalmitis infections have been reported which was related to *C. glabrata*. *C. glabrata* endophthalmitis following penetrating keratoplasty is very rare but has devastating effects. It typically occurs within the first and second week post penetrating keratoplasty (Muzaliha *et al*., 2010). In contrast, there were cases occurred as early as 10 hours post transplantation or as late as 5 months post-surgery (Al-Assiri *et al.*, 2006; Muzaliha *et al*., 2010).

2.5 Nosocomial infections caused by *C. glabrata*

Vazquez *et al*. (1998) reported that patients infected with *C. glabrata* had a longer duration of hospitalization (18.8 and 7.6 days, respectively; $P < 0.001$) prior to antimicrobial use (100 and 65%, respectively; $P < 0.001$) compared to patients from whom *Candida* species were not recovered during the study. The spread of nosocomial infection of *C. glabrata* requires a complex interaction of environmental and human reservoirs, similar with those caused by *C. albicans* (Hunter, 1991; Vazquez *et al*., 1993). Two studies have implicated carriage on the hands of hospital personnel as a possible source of an outbreak (Baradkar *et al.*, 2009).

Previous understanding of the pathogenesis of *C. glabrata* colonization and infection assumed that the organisms responsible for disease were endogenously acquired from the patients' own flora (Fidel *et al.*, 1999), whereby patients carrying the same strain type of *C. glabrata* over time (Rodrigues *et al.*, 2014), with minimal strain diversity among individual patients (Rodrigues *et al*., 2014). In that study, it is also noted that 71% of patients with positive *C. glabrata* cultures had more than

one *Candida* species isolated. Studies are needed to define the reservoirs of infection, and the mode of transfer and measures for preventing the spread of infection.

2.6 Cultural and biochemical tests for identification of *C. glabrata*

On Sabouraud's dextrose agar, *C. glabrata* forms glistening, smooth, cream-colored colonies which are relatively indistinguishable from those of other *Candida* species except for their relative size, which is quite small (Macêdo *et al.*, 2008). CHROMagar is relatively a new agar medium which distinguishes different *Candida* species by color. As a result of biochemical reactions, *C. glabrata* colonies appear pink to purple, in contrast to *C. albicans* colonies, which appear green to blue-green (Fidel *et al*., 1999). Figure 2.2 showed the colour of different *Candida* species on CHROMagar medium. According to the recent studies, *C. glabrata* and two newly described yeasts (*C. bracarensis* and *C. nivariensis*) produce white colonies on CHROMagar medium (Bishop *et al.*, 2008). In contrast to *C. albicans*, which ferments and/or assimilates a number of sugars, *C. glabrata* ferments and assimilates only glucose and trehalose (Kwon & Bennet, 1992).

Figure 2.2: Colony colour of different species of *Candida* on a Chromagar plate.

a, *C. albicans;* b, *C. dubliniensis;* c, *C. tropicalis;* d, *C. glabrata;* and e, *C. krusei.* (Shettar *et al*., 2012)

The feature has been used by several commercially available kits (API 20C, Uni-Yeast-Tek, and YeastIdent) to identify yeast to the level of genus and species. Other rapid yeast identification methods are commercially available kits such as Vitek (bioMérieux Vitek), Yeast Star (CLARC Laboratories, Heerlen, The Netherlands), API Candida (bioMérieux, France) (Verweij *et al*., 1999), GLABRATA RTT (Fumouze Diagnostics) and Glabrata Quick (Hardy Diagnostics) (Freydiere *et al*., 2003; Fraser *et al*., 2012). These kits have been designed based on sugar utilization activity of yeasts. The sugars are coated inside the wells of a test tray and the result should be observed after incubation.

Trehalase is a glycoside hydrolase enzyme that catalyses the conversion of trehalose to glucose. *C. glabrata* has the ability to ferment trehalose and change the pH indicator from red to yellow (Rodrigues *et al.,* 2014). For example, GlabrataQuick™ Trehalose Fermentation Kit (Hardy Diagnostics, USA) is designed for rapid detection of trehalase. The kit utilizes three groups of carbohydrates trehalose, maltose and sucrose for testing (Figure 2.3).

Although *C. nivariensis* and *C. bracarensis* share all the phenotypic characteristics of *C. glabrata*, the trehalose fermentation of *C. nivariensis* and *C. bracarensis* are rather weak. *C. nivariensis* displays positive trehalose fermentation only after 7 to 8 days of incubation, while *C. bracarensis* has very weak threhalose activity (Wahyuningsih *et al.,* 2008).

Figure 2.3: Result of Hardy Diagnostics GlabrataQuick rapid test.

2.7 Pseudohyphae production

Under specific environmental conditions (temperature, energy, organic nitrogen, pH, oxygen, osmotic potential, minerals such as calcium etc), some fungal species may switch from spheres (yeast cells) to elongated conjoined buds (pseudo hyphae) or to filamentous (hyphae) (Fidel *et al*., 1999). Production of pseudohyphae in laboratory is usually performed by culturing the yeast on a Cornmeal agar and the yeast growth was observed microscopically. *C. glabrata* is negative for pseudohyphae production. This pathogenic yeast normally produces budding yeast-like cells of 2.0-4.0 x 3.0-5.5 μm in size without pseudohyphae (Brown & Gow, 1999; Rodrigues *et al*., 2014). *C. bracarensis* does not have the ability to produce pseudomycelium and *C. nivariensis* is practically unable to form pseudohyphae or true hyphae on corn meal agar (Bishop *et al.*, 2008).

2.8 Molecular approaches for identification and strain typing of *C. glabrata*

Several molecular biology techniques have been developed to differentiate medically important *Candida* species. These newer methods include restriction fragment length polymorphisms (RFLP), pulsed-field gel electrophoresis, randomly amplified polymorphic DNA (RAPD), and DNA probes (Jordan, 1994, Khattak *et al*., 1992, Lockhart *et al.*, 1997, Vazquez *et al.*, 1998).

C. glabrata have been separated to 28 strain types based on the different chromosomal molecular weights using electrophoretic karyotyping (EK) technique (Khattak *et al*., 1992; Vazquez *et al.*, 1998). Additionally, definite identification of *C. glabrata,* can be performed using molecular techniques such as multiplex PCR targeting the internal transcribed spacer region (ITS1–ITS2 & ITS3–ITS4), peptide nucleic acid fluorescence *in situ* hybridization method, RFLP analysis or sequencing of ITS and 26S rRNA D1D2 gene regions (Bishop *et al*., 2008; Leaw *et al*., 2006).

2.8.1 Polymerase chain reaction (PCR) for identification of *C. glabrata, C. nivariensis* and *C. bracarensis*

Rapid identification of bacteria in clinical samples is important for identification of microorganism. Many primer sets have been developed to detect species-specific sequences using simple PCR assays (Mitterer & Shmidt, 2006). Enache-Angoulvant *et al.* (2011) developed a PCR-based assay using a single primer pair targeting the *RPL31*gene, a gene encoding for a protein component of the large (60S) ribosomal subunit to allow discrimination between *C. glabrata*, *C. bracarensis*, and *C. nivariensis* according to the size of the amplicons, i.e., 1,061 bp for *C. glabrata*, 902 bp for *C. bracarensis*, and 665 bp for *C. nivariensis.* The development of such assay is expected to improve our knowledge on the respective epidemiology and pathogenic importance of these three species.

2.9 Strain typing

High degree of genetic diversity among *C. glabrata* isolates is due to adaptation of the yeast in different environment including temperature changing, presence of antifungal agent, poor diet and other situations. Therefore the genotypic characteristic of each *C. glabrata* strain could be unique and different (Jandric & Schuller, 2011; Rodrigues *et al*., 2014; Zubko & Zubko, 2014).

The random amplified polymorphic DNA (RAPD) technique relies on the use of arbitrary primers which are annealed to genomic DNA using low temperature conditions. Priming at a number of closely adjacent complementary sites allows the subsequent amplification of dispersed genomic sequences by *Taq* DNA polymerase enzyme. This technique detects genetic polymorphisms and does not depend on prior knowledge of species-specific sequences (Valério *et al.,* 2006). The core sequence of phage M13 and two synthetic oligonucleotides, $(GACA)_4$, and $(GTG)_5$ are usually used

as primers in minisatellite-primed PCR experiments to identify genomic varieties of common fungi species (Roque *et al.,* 2006). Strain typing of *Candida glabrata* isolates has been performed using randomly amplified polymorphic DNA (RAPD) technique in Poland. *C. glabrata* strains were allocated into four genetic similarity groups (d-g) and six unique profiles were distinguished (a-c, h-j) using HP1247, CD16AS, ERIC-2, OPE-3 and OPE-18 primers, respectively (Paluchowska *et al*., 2014).

2.10 Treatment and antifungal susceptibility of *Candida glabrata*

Amphotericin B has been the "gold standard" for treatment of systemic fungal infections including candidemia, despite having a high adverse effect profile. Amphotericin B resistance has not been described in *C. glabrata* (Gallis *et al*., 1990), although the MICs are higher than those seen for *C. albicans, C. glabrata* isolates tend to be associated with higher MICs for all azoles (Vazquez *et al.,* 1998; Vazquez *et al.,* 1991). Table 2.1 shows the general susceptibility of *Candida* species as documented in the USDA document (2011). *C. glabrata* is susceptible to flucytosine, echinocandin and amphotericin but demonstrate reduced susceptibility to azoles including fluconazole, itraconazole, voriconazole, and posaconazole.

According to several investigators, the increase in the frequency of *C. glabrata* infections has paralleled the increase use of fluconazole in some hospitals (Abi-Said, 19971; Wingard et *al*., 1993; Wingard, 1994). Fluconazole-resistant isolates have been found predominantly in AIDS patients with OPC and esophageal candidiasis and fungemic patients and among vaginal isolates (Fidel *et al.*, 1999). About 68 % of isolates in San Antonio, Texas were resistant to the fluconazole (Esfandiary *et al*., 2012; Li *et al*., 2007a).

C. glabrata vaginitis has been moderately successfully treated with boric acid, but this is not curative in one third of patients (Guaschino *et al*., 2001; Sobel & Chaim, 1997;

Sobel, 2007; Sobel *et al*., 2003). Amphotericin B suppositories in patients infected with non-*albicans Candida* spp. were studied by Phillips and found to be promising; however, symptomatic *C. glabrata* vaginitis is often unresponsive to these regimens (Khan *et al*., 2008; Phillips, 2005). Clotrimazole has been widely used in the treatment and prevention of OPC in the growing immunocompromised population (Pelletier *et al*., 2000). Clotrimazole vaginal tablets have produced cure rates comparable with those of nystatin vaginal tablets for treatment of vaginal candidiasis (Sawyer *et al*., 1975). Despite the extensive usage of clotrimazole in HIV-infected patients, little is known about the emergence of microbial resistance to this topically administered imidazole in *C. glabrata*.

Table 2.1: General pattern of susceptibility of *Candida* species (Source: USDA, 2011)

Table 3. General patterns of susceptibility of *Candida* species.

Species	Fluconazole	Itraconazole	Voriconazole	Posaconazole	Flucytosine	Amphotericin B	Candins
Candida albicans	S	S	S	S	S	S	S
Candida tropicalis	S	S	S	S	S	S	S
Candida parapsilosis	S	S	S	S	S	S	S to R[a]
Candida glabrata	S-DD to R	S-DD to R	S-DD to R	S-DD to R	S	S to I	S
Candida krusei	R	S-DD to R	S	S	I to R	S to I	S
Candida lusitaniae	S	S	S	S	S	S to R	S

NOTE. I, intermediately susceptible; R, resistant; S, susceptible; S-DD: susceptible dose-dependent.

[a] Echinocandin resistance among *C. parapsilosis* isolates is uncommon.

There are so many well-established methods for antifungal resistance testing in microbiology laboratories around the world such as E-test and microdilution test (www.biomerieux-usa.com). E-test consists of a predefined gradient of antibiotic concentrations on a plastic strip and is used to determine the Minimum Inhibitory Concentration (MIC) of antifungal agents. The yeast culture is first streaked across the surface of a RPMI 1640 agar plate then E-test strips (fluconazole, voriconazole,

posaconazole, isavuconazole, amphotericin B, and caspofungin) are placed onto the surface and incubated for 24-48h (Pfaller *et al.,* 2001).

Microbroth dilution method for antifungal susceptibility testing can be performed with different fungal drug concentrations distributing in the wells of microtiter plates using RPMI 1640 medium. The MICs can be determined according to the approved standard Clinical and Laboratory Standards Institute (CLSI) document M27-A3 (2008).

2.11 Virulence factors of *C. glabrata*

Virulence factors are those attributes of an infectious microorganism that permit it to actively breach host defences that ordinarily restrict invasive growth of other microbes (Cole, 2003). Unlike *C. albicans* which demonstrates morphological flexibility (Sudbery, 2011), the pathogenicity of *C. glabrata* seems to be independent of morphology. Additionally, *C. glabrata* does not cause extensive epithelial damage, probably due to its lack of an invasive growth form (Brunke & Hube, 2013). Nevertheless, both fungi are similarly successful as commensals and as pathogens (Brunke & Hube, 2013). According to the review of Brunke & Hube (2013), *C. albicans* follows a strategy which can be described as 'shock and awe' as it actively invades epithelia when the circumstances permit and elicits strong immune responses. Macro- and micronutrients from damaged host tissue are taken up by a broad range of acquisition systems, and the defending macrophages may be killed by the formation of hyphae. Tissue damage by the fungus and the activated immune system can lead to severe disease, and death (Coco *et al*., 2008; Redding *et al*., 2002). In contrast, *C. glabrata* is believed to rely on autophagy and some so far uncharacterized nutrient uptake mechanisms for nutrient supply, but would not elicit rapid tissue damage to release nutrients from host cells. It is speculated that once *C. glabrata* reaches internal

organs, its inherently high resistance to many commonly used antifungals makes treatment more problematic (Brunke & Hube, 2013) (Figure 2.4).

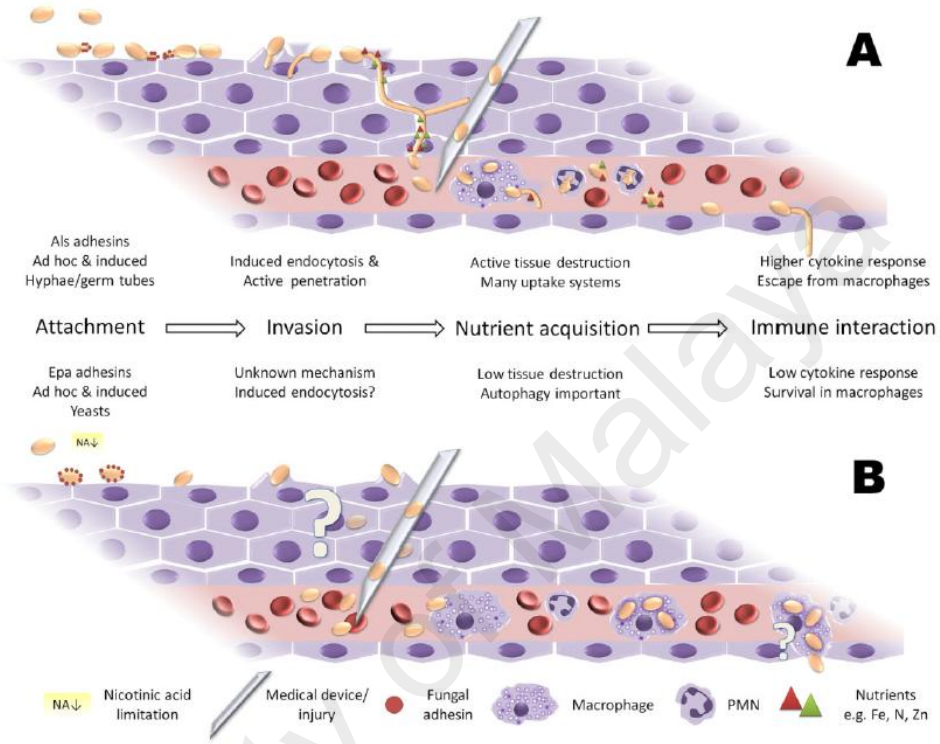


Figure 2.4: Schematic overview of the two different infection strategies of *C. albicans* and *C. glabrata*. A. *C. albicans* form hyphae & aggressively destroys tissue, eliciting a strong immune response. B. Many aspects of *C. glabrata* pathogenicity are still unknown, like the precise mechanism of invasion. Active host tissue damage is low, as is the immune response. (Source: Brunke and Hube, 2013).

2.12 Proteinases and other hydrolytic enzymes

Proteinase production by *Candida* species has been associated with pathogenicity (Fidel *et al.*, 1999). For example, virulent *C. albicans* isolates often produce aspartyl proteinase, and thus are more pathogenic in a variety of animal models (Fidel *et al.*, 1999). Historically *C. glabrata* isolates have been known to be capable of proteinase production; however the type of proteinase was not specified (Li *et al.*, 2007b). Recently researchers discovered production of extracellular hydrolyse enzymes

in *C. glabrata*, including serine-, cysteine-, metalo- and aspartyl- proteinases (García *et al*., 2009; Li *et al*., 2007b). Esfandiary *et al.* (2012) reported that 41% of *C. glabrata* isolates from blood infections in their study are able to produce phospholipases, indicating the importance of this enzyme in causing infections.

2.13 Biofilm formation

Biofilm is heterogeneous organized communities of fungal cells which is produced from matrix of extracellular polymeric substances and on the surfaces of medical devices (Jin *et. al.,* 2004). According to previous studies, all non-*albicans Candida* species are able to produce biofilms (Silva *et al.,* 2009). The production of biofilms (Zadik *et al.,* 2010) in *C. glabrata* is less intensive in comparison with *C. parapsilosis* and *C. tropicalis*. Based on data from scanning electron microscopy (SEM), the reason of this difference is probably due to the extracellular matrix of these fungal species (Silva *et al.,* 2009). Interestingly, in *C. glabrata* cell matrix, the level of both protein and carbohydrates is high (Silva *et al*., 2009). Hence, *C. glabrata* is believed to have the ability to form biofilm and this property may increase with the presence of higher serum levels and inflammatory factors (Esfandiary *et al.,* 2012; Mundy & Cormack, 2009).

CHAPTER 3

MATERIAL AND METHODS

3.1 Identification of *C. glabrata* isolates

3.1.1 Yeast isolates

In this study, 185 *C. glabrata* isolates collected from various clinical specimens from the Diagnostic Microbiology Laboratory, University of Malaya Medical Centre (UMMC), Kuala Lumpur, Malaysia, from 2008-2012 were investigated in this study. These isolates had been previously identified as *C. glabrata* using routine carbohydrate assimilation tests, as described by Ng *et al*., (2001). The isolates had been recovered from vaginal swabs (n= 142), blood culture (n=11), urine (n=14), respiratory secretions (n=4) and others (n=14). The isolates were stored in 20% glycerol stock solution at -80^{o}C prior to use. To subculture *Candida spp*., the isolates were taken from 20% glycerol stock using a sterile standard size wire loop and inoculated onto potato dextrose agar (PDA) plates (Appendix A). The cultures were incubated at 30ºC for 18 to 24 hours. Two reference strains i.e., *C. albicans* ATCC90028 and *C. parapsilosis* ATCC 22019 were also included in this study.

3.1.2 Phenotypic characterisation of *C. glabrata* and *C. nivariensis*

The isolates were subcultured onto both PDA and Chromogenic Chromagar *Candida*® agar (Difco, USA) at 37 °C for up to 2 days (Appendix A). The colony morphology was observed and the colour change on Chromagar was recorded. API 20C AUX tests (bioMérieux, Marcy l'Etoile, France) were used for biochemical identification of randomly selected isolates. GlabrataQuick™ Trehalose Fermentation Kit (Hardy Diagnostics, USA) which is designed for rapid detection of trehalase, was

performed in accordance to the instruction manual of the manufacturer. Enzymic profiling of 6 *C. glabrata* and 2 *C. nivariensis* selected isolates was determined using API ZYM kit (bioMerieux, Marcy I'Etoile, France). Egg-yolk agar plate methods were used to assess the phospholipase activities of the isolates (Price *et al*., 1982).

3.1.3 Growth reaction on Chromagar

To prepare the agar, 6.24 g of Chromogenic Chromagar *Candida*® agar (Difco, USA) powder was dissolved in 200 ml of distilled water. The solution was mixed well with frequent agitation for 3 to 5 minutes. The solution was then boiled until all powder was dissolved. The agar was cooled to 45ºC before pouring it into sterile Petri dishes. Each yeast isolate was subcultured onto the agar and incubated at 37Cº for 2 days. The colony colours were recorded (Appendix A).

3.1.4 API AUC tests

API 20C AUX tests (bioMérieux, Marcy l'Etoile, France) were used for phenotypic identification of the isolates according to the instructions of the manufacturer. Several colonies of a 24h-old culture were suspended in the API C medium (provided in the kit) and the density of the yeast suspension was adjusted to 2 McFarland. 100 µl of each suspension was added into respective cupule and incubated at 37Cº for 48h. Five millilitres of distilled water was distributed into the tray (provided by the kit) to create the humid atmosphere. After incubation at 30ºC for 24 to 72 hours, the turbidity of all the cupules were checked and compared to the first cupule (negative control). The numerical profiles of each isolate were determined on the result sheets. The numbers (1, 2, 4) indicated in each group were added according to the results obtained. The profile obtained was entered into API database to search for matching profile.

3.1.5 Glabrata Quick Kit test

GlabrataQuick™ Trehalose Fermentation Kit (Hardy Diagnostics, USA) which is designed for rapid detection of trehalase, was used in this study. 26 *C. glabrata* and two *C. nivariensis* isolates were subjected to the tests. The kit identifies *C. glabrata* based on the results of sugar (trehalose, maltose and sucrose) assimilation. Heavy suspension of each isolate was prepared in sterile inoculation and inoculated into buffer tubs that were prepared inside the kit, prior to incubation at 35ºC for 1-2 h. A change of colour from blue or blue-green to yellow or greenish-yellow for the carbohydrate wells (rows C, D and E) is considered positive. The reference well (A) remains negative (blue or blue-green) throughout the incubation. *C. glabrata* produces positive results with trehalose, but negative with maltose and sucrose (Figure 4.4).

3.2 Molecular identification *C. nivariensis*

3.2.1 DNA extraction

Genomic DNA was extracted from all the yeast isolates using MasterPure™ Yeast DNA Purification Kit (EpiCenter, Madison, WI). A half loop of yeast colony from a PDA plate was suspended in 300 µl yeast cell Lysis solution in a microcentrifuge tube. The cell suspension was mixed thoroughly using a vortex and incubated at 65 °C for 15 min. The cell lysate was then placed on ice for 5 min and after that 150 µl MPC protein precipitation Reagent (provided in the kit) were added to the mixture. The solution was centrifuged at 15,000 rpm for 10 min. The supernatant (400 µl) was then transferred to a clean microcentrifuge tube. Yeast DNA was precipitated using 500 µl isopropanol followed by centrifugation at 15,000 rpm for 10 min. The pellet was washed with 0.5 ml 70% ethanol and suspended in 30 µl sterile distilled water. The DNA solution was used as template for PCR assays. The DNA purity and concentration were measured using a nanophotometer (Implen, Munich, Germany).

3.2.2 Polymerase chain reaction assay for discrimination of *C. glabrata, C. nivariensis* and *C. bracarensis* (Enache-Angoulvant *et al.*, 2011)

The yeast DNA was amplified using a singleplex PCR assay for discrimination between *C. glabrata, C. bracarensis,* and *C. nivariensis,* as described by Enache-Angoulvant *et al.*, (2011). Polymerase chain reaction (PCR) was performed in a final volume of 25 µl containing 2 µl DNA, 0.5 µl *Taq* polymerase (5 U/µl) (Fermentas, Lithuania), 0.5 µl deoxyribonucleoside triphosphate mix (10 mM of each nucleotide), 2.5µl 10X PCR buffer, 1.5µl $MgCl_2$ and 0.125 µl 10 mM of each primer RPL31A (5´-GCCGGTTTGAAGGACGTTGTTACT-3´) and RPL31B (5´-GAACAATGGGTTCTTGGCGT-3´) in a Veriti^{Tm} thermal cycler (Applied Biosystems, USA) (Enache-Angoulvant *et al.*, 2011) (Table 3.1). A touch-down amplification program involved an initial cycle 3 min at 95 °C, followed by 3 cycles with 1 cycle consisting of 30 s at 95 °C, 30 s at 62 °C, and 30 s at 72 °C ; 3 cycle with 1 cycle consisting of 30 s at 95 °C, 30 s at 58 °C, and 30 s at 72 °C; 3 cycle with 1 cycle consisting of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C; 28 cycle with 1 cycle consisting of 30 s at 95 °C, 30 s at 50 °C, and 30 s at 72 °C; and a final extension step of 10 min at 72 °C (Enache-Angoulvant *et al.*, 2011). The amplified products were analyzed by electrophoresis on a 1% (w/v) ethidium bromide-stained agarose gel (Appendix A) at 100 V for 40 min, and the image was captured using InGenius gel documentation system (Syngene, England). The PCR assay was interpreted based on the presence of amplified products on agarose gel: *C. glabrata (*1061 bp), *C. nivariensis* (665 bp) and *C. bracarensis* (902 bp).

Table 3.1: Composition of PCR reagents

Reagent	Stock concentration	Volume (µl per reaction)
DNA template		2 µl
Forward Primer	100 pmol/µl	0.125µl
Reverse primer	100 pmol/µl	0.125µl
dNTPs	10 mM	0.5 µl
Taq polymerase	5U/µl	0.5 µl
PCR buffer	10 X	2.5 µl
$MgCl_2$	25 mM	1.5 µl
dH_2O		17.75µl
Total volume		25

3.2.3 Agarose gel electrophoresis

Four microliters of PCR products were added with 2 µl loading dye (Fermentas, Lithuania) before loading into a well in an agarose gel (1% in Tris-borate-EDTA [TBE] buffer) pre-stained with ethidium bromide (0.5 µg/ml) (Appendix A). A DNA Ladder VC 100bp Plus (Vivantis, USA) was included in every run. The electrophoresis was performed in 1X Tris-Base-EDTA (TBE) buffer at 90V for 60 min. The DNA bands were visualized and photographed under ultraviolet light using InGenius Gel Documentation System (Syngene, United Kingdom).

3.2.4 Amplification and sequence analysis of the internal transcribed spacer (ITS) region and the 26S rRNA gene D1D2 domain of isolates

For amplification of the internal transcribed spacer (ITS) region and the 26S rRNA gene D1D2 domain of yeast isolates, primers ITS1 (5'-GTC GTA ACA AGG TTT CCG TAG GTG-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (White

et al., 1990) and primers NL1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3') and NL4 (5'-GGT CCG TGT TTC AAG ACG G-3') (Kurtzman *et al*., 1998) were used. The amplicons were purified and sequenced using forward and reverse primers.

3.2.5 Purification of PCR products

The amplification products were purified using a QIAquick PCR Purification Kit (USA) in accordance to the manufacturer's instructions. The PCR product (20 µl) was first transferred to a 1.5 ml microcentrifuge tube. Five volumes of PB Buffer (provided in the kit) was added to 1 volume of the PCR product and mixed by vortexing. The QIAquick spin column was then placed in a 2 ml collection tube. The sample mixture was added into the QIAquick spin column and centrifuged at full speed (approximately 13,000 rpm) for 30 s. The flow through was then discarded and the QIAquick spin column was returned to the 2 ml collection tube. A total of 750 µl wash buffer PE was added into QIAquick spin column and left at room temperature for 1 minute. The column was then centrifuged at full speed (approximately 13,000 rpm) for 60s. The flow through was discarded and the QIAquick column was returned to the same tube. The column was then centrifuged for an additional 1 min at maximum speed to make it fully dry. The dried QIAquick column was then transferred to a new 1.5 ml microcentrifuge tube and 20 µl of elution buffer (provided in the kit) was added into the centre of the column. The column was then left at room temperature for 1 min to ensure that the elution buffer had been absorbed by the matrix properly. Finally, it was centrifuged for 1 min at full speed to elute the purified DNA. The purified DNA was stored at -20 °C prior to sequence determination.

3.2.6 Sequence determination and analysis

Sequence determination of PCR products was performed using the service provided by Firstbase Laboratory, Shah Alam Malaysia. The amplified DNA fragments were sequenced using Big Dye® Terminator Cycle sequencing kits (Applied Biosystems, USA) in an ABI-3730 Genetic Analyzer (Applied Biosystems, USA). PCR primers were used as sequencing primers. The sequences obtained were assembled using Geneious Pro 4.7.6 and analysed with nucleotide-nucleotide BLAST (BLASTN) program (http;//blast.ncbi.nlm.nih.gov/Blast).

To determine the sequence similarity of the *C. glabrata* isolates with others from different geographical regions, ITS1 sequences were assembled and the neighbor-joining method in MEGA version 5.2 was used to determine the phylogenetic status of the isolates (Tamura *et al*., 20011) The reliability of different phylogenetic groupings was evaluated using bootstrap tests (1000 bootstrap replications).

3.3. Molecular typing of *C. glabrata* isolates using random amplification polymorphic DNA analysis (RAPD)

PCR fingerprinting technique was performed to determine the genetic relatedness amongst the yeast isolates investigated in this study. Amplification was carried out in volumes of 50 µl containing 1µl (25 ng) yeast DNA as prepared in section 3.2.1, 0.25 µl *Taq* polymerase (5 U/ µl) (Fermentas, Lithuania), 1 µl deoxyribonucleoside triphosphate mix (10 mM of each nucleotide), 5 µl of 10X PCR buffer, 6 µl $MgCl_2$ (25 mM) and 0.25 µl 100 pmol/µl of each primer M13 (5-GAGGGTGGCGGTTCT 3-) and $(GTG)_5$ (5- GTGGTGGTGGTGGTG 3-) (Meyer *et al*., 1993) in Veriti^{Tm} thermal cycler (Applied Biosystems, USA). The composition of the reaction mixture is shown in Table 3.2. The PCR condition used were: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 20 sec,

annealing at 50°C for 1 min and extension at 72°C for 20 sec. The PCR were continued with a final extension at 72°C for 6 min (Meyer *et al.,* 1993).

Table 3.2: **Preparation of reaction mixture for RAPD PCR assay for amplification of *C. glabrata* and *C. nivariensis* genome with primers M13 and $(GTG)_5$**

Reagent	Stock Concentration	Volume (µl per reaction)	Final concentration
Primer	100 pmol/µl	0.25	25 pmol
dNTPs	10 mM	1	200 µM
Taq polymerase	5U/µl	0.5	2.5 U
PCR buffer	10 X	5	1 X
$MgCl_2$	25 mM	6	50 mM
DNA template		1	25 ng/µl
dH_2O		36.25	
Total volume			50

3.3.1 Agarose gel electrophoresis

A volume of 10 µl of PCR was mixed with 2 µl of 6X loading dye solution (Fermentas, USA) prior to electrophoresis on a 2% agarose gel at 100V for 2.30 h. The 100 bp DNA marker (Vivantis, Malaysia) was included to enable size estimation of the PCR products. The gel was then visualized and photographed under UV light for digital record using AlphaDigiDoc^{TM} System (Alpha Innotech, USA).

3.3.2 DNA fingerprinting Analysis using GelCompar 2.0

DNA fingerprinting profile for each isolate was carefully analysed to identify a common pattern among the isolates. Based on the fragment-for-fragment (Pairwise) comparisons, the categories of relatedness of other patterns were assigned in reference

to the common pattern. The interpretive criteria as recommended by Tenover *et al*. (1995) were used to ascertain the relatedness of each isolate. Isolates with indistinguishable DNA pattern were considered genetically related. Isolates were considered closely related if the RAPD pattern was changed by a single genetic event such as insertion, deletion and point mutation that resulted in two to three bands difference with the common pattern (Tenover *et al.*, 1995). Other isolates that differed by ≥ 4 up to 6 band patterns to the common patterns were considered possibly related. For isolates with patterns that differed by more than seven bands were considered unrelated. The criteria that were considered for interpretation are shown in Table 3.3.

Table 3.3: Criteria for interpreting RAPD patterns (Tenover *et al.*, 1995).

Categories of relatedness	No. of bands differences
Indistinguishable	0
Closely related	2-3
Possibly related	4-6
Different	≥7

The RAPD photograph was scanned to be analysed using the Gel Compar II Version 4.0 software package (Applied Maths, Kortrijk, Belgium). The program compares the motilities and the number of DNA fragments by pairwise comparison of all the isolates on the gel. The degree of similarity among the DNA patterns generated was scored by Dice coefficient of similarity (F) to give the proportion value of the shared DNA fragments among the isolates. The following formula was used to calculate the F value in this program.

$F = \frac{2n_{xy}}{n_x + n_y}$

n_x = the total number of DNA fragment from isolates x

n_y = the total number of DNA fragment from isolate y

n_{xy} = the number of DNA fragment from isolate shared among the two isolates

Two identical DNA fragment length patterns were indicated by the maximum F value (F=1). The overall similarities based on the F values of the isolates were summarized in dendrogram in which the similarities were shown in percentages. The steps involved in the analysis of the fingerprinting profiles are shown below:

(1)Creating and configuring a new database

Creating a new database

Creating a new experiment

Adding a new gel in an fingerprint group

(2)Processing gels

Opening the gel and defining densitometric curves

Normalizing the 1st gel of the fingerprinting type

Adding lanes to the data base

(3) Selecting entries for the cluster analysis

Manual selection of entries

Automatic selection of entries

Creating and upgrading a selection

(4)Comparing entries/cluster analysis

Pair-wise comparison between two entries

Comparison between more than two entries

Calculating a dendrogram

(5) Display functions

Group functions

Matrix display functions

(6) Printing a cluster analysis

3.4 Determination of antifungal susceptibilities of yeast isolates

3.4.1 E-test Method

E-tests were performed using amphotericin B, fluconazole, caspofungin and voriconazole strips (Biomerieux, France) in accordance to the manufacturer's instructions. RPMI 1640 medium with 2% glucose buffered with 3-(N-Morpholino) propanesulfonic acid 4-Morpholinepropanesulfonic acid (MOPS) and 1.5% Bacto agar was used to prepare the E-test RPMI-agar plates (Appendix A). The inoculum suspension was prepared by suspending half of the colony of a 48 hour-old yeast culture into 5 ml of sterile normal saline (Appendix A). The turbidity of the yeast suspension was then adjusted to 0.5 McFarland standards. A sterile swab was soaked into the suspension and squeezed against the wall of the test tube, before used to streak the entire agar surface in three directions to distribute the inoculum evenly. The agar plate was then left to dry before the application of the strips, using an E-test applicator (vacuumpen, AB bioMerieux). Etest were performed by positioning two strips on a 90 mm agar plate (CLSI, 2008) and incubated at 35 °C for 24, 48 and 72 hours. The MIC was read where the border of the elliptical inhibition zone intersected the scale on an antifungal strip. For MIC testing of amphotericin B and caspofungin, the endpoint was read where there was 100% inhibition of growth and for itraconazole, voriconazole and fluconazole (the azoles), the endpoint was read at 80% inhibition of growth.

Quality control strains (Table 3.4) were used whenever new batch of agar medium were prepared. Categorization of *C. glabrata* as susceptible or resistant strains was based on the interpretative criteria published by previous studies (Nguyen *et al.*, 1998; CLSI, 2008; Rex *et al.*, 1997) in Table 3.5.

Table 3.4: Quality control strains used in E-test method (Source: E-test, Biomerieux)

Drugs	*C. parapsilosis* ATCC 22019 (µg/ml)	*C. albicans* ATCC 90028 (µg/ml)
Amphotericin B	0.25 - 1	0.125 - 0.5
Caspofungin	0.25 - 2	0.064 - 0.25
Itraconazole	0.064 - 0.25	0.064 - 0.25
Voriconazole	0.016 - 0.064	0.004 - 0.016
Fluconazole	1 – 8	0.125 – 0.5

Table 3.5: The interpretation guide for *Candida* species, according to CLSI for E-test (Source: E-test, Biomerieux)

Antifungal MIC (range, µg/ml)	Interpretive criteria MIC (µg/ml)	Quality control strain MIC range (µg/ml)
Amphotericin B (0.002-32)	No conclusive breakpoints*	0.125-.05
Caspofungin (0.002-32)	S, ≤ 2	0.064-0.25
Fluconazole (0.016-256)	S, ≤ 8; S-DD: 16-32, R, ≥ 64	0.125-0.5
Voriconazole (0.002-32)	S, ≤ 1; S-DD, 2; R, ≥ 4	0.004-0.016

S, Susceptible; S-DD, susceptible dose-dependent, R, resistant

C. albicans ATCC 90028 was used as the quality control strain.

*Note: Isolates with MIC of >1 µg/ml (Nguyen *et al.,* 1998) were considered to be resistant to Amphotericin B.

3.4.2 Microbroth dilution method for MIC determination of clotrimazole

For MIC determination of clotrimazole, the microbroth dilution method was performed according to the recommendations of CLSI (2008). A stock solution of 25mg/ml Clotrimazole (MERCK, USA) was prepared in dimethylsulphoxide (DMSO). RPMI 1640 medium supplemented with glucose and MOPS (Appendix A) was used to make serial dilution of the drug. A 96-well-microtiter plate (NUNC, Denmark) were inoculated with 100 µl of the serially-diluted clotrimazole solution, ranging from 0.03 and 16 µg/ml.

The inoculum suspensions were prepared by picking five colonies of 1 mm in diameter from 24-hour-old cultures of *C. glabrata*. The colonies were suspended in 2 ml of distilled water. The resulting suspension was vortexed for 15 seconds and adjusted to 75% to 85% transmittance using a spectrophotometer (Genesys, USA). The yeast suspension (50 µl) was added to a tube containing 4.95 ml distilled water (1:50 dilution). This suspension was used as stock suspension. A working suspension was prepared by a 1:20 dilution of the stock suspension with RPMI 1640 medium, which resulted in 5 x 10^2 to 2.5 x 10^3 cells per ml. The microtiter plate was incubated at 37 °C and the presence or absence of visible growth was observed with a reflecting mirror after 24 h and 48 h of incubation. The MIC endpoint was determined as the lowest drug concentration exhibiting approximately 80% reduction of growth compared with the control growth for azole antifungal agent. Quality control organisms (*C. parapsilosis* ATCC 22012, *C. albicans* ATCC 90028 and *C. krusei* ATCC 6258) were included in this study. Although there are no established interpretive breakpoint criteria to designate a *Candida* isolate as either susceptible or resistant to clotrimazole, Pelletier *et al.* (2000) suggested clotrimazole MIC of ≥0.5 µg/ml as a possible interpretive resistance breakpoint.

3.5 Investigation of enzyme production and biofilm formation of *C. glabrata* isolates

3.5.1 Phospholipase activity

Extracellular phospholipase activity of *C. glabrata* was measured by using the egg-yolk agar plate method of Price *et al.* (1982) with slight modification (Appendix A). Two eggs were sterilised for 1 h with 95% alcohol and egg yolk was obtained using aseptic technique. To prepare the egg-yolk agar, ten milliliters of egg yolk was added to an equal volume of sterile distilled water in a Falcon tube. The tube was then inverted several times to prepare a homogenous suspension. The egg yolk suspension was then incorporated into a sterilized Sabouraud's dextrose agar (SDA) medium added with 1 M sodium chloride, and 0.005 M calcium chloride (Appendix A). A loopful of an overnight yeast culture was inoculated onto the egg-yolk agar plate using a sterilized flamed loop. Each isolate was incubated at 37 °C for 7 days. The agar was examined daily for formation of hazy zone around the colony. The diameter of colony (a) and the diameter of hazy zone around the colony (b) were measured. Phospholipase production of isolates were calculated as P_z=a/b, as described by (Price *et al*., 1982). *C. albicans* (ATCC 90028) was used as a positive control.

3.5.2 APIZYM analysis

A total of 8 clinical isolates of *C. glabrata* and 2 isolates of *C. nivariensis* were examined for enzymic production using an APIZYM kit (Biomerieux, France). Yeast isolates were subcultured on Potato Dextrose agar (PDA) and incubated overnight at 37 °C. A loopful of yeast culture was suspended in API suspension medium (provided in the kit) and adjusted to 5-6 McFarland, as recommended by the manufacturer (bioMerieux, France). Five milliliters of sterile distilled water was added to an

incubation box, consisting of a plastic tray with honey combed well and lid to create a humid atmosphere. APIZYM strip was removed from its packaging and placed in the incubation box. A volume of sixty-five microliters of the yeast inoculum was dispensed into each of the APIZYM strip cupules. Table 3.6 shows the enzymes tested in the APIZYM system and the respective substrates used by these enzymes. After inoculation, the plastic lid was placed on the tray and incubated at 37 °C for 4 hours. After incubation, 1 drop each of Zym A and Zym B reagent was added to each cupule. The colour was allowed to develop for 5 min and any colour changes were recorded. The colour scale given by the manufacturer ranged from 0 (negative reaction) to 5 (maximum positive reaction), where 1 corresponded to 5 nmols, 2 to 10 nmols, 3 to 20 nmols, 4 to 30 nmols and 5 to 40 nmols of each APIZYM substrate metabolized by the isolates. *C. albicans* ATCC 90028 was used as a quality control strain in this test.

Table 3.6 enzyme and the respective substrates used in the APIZYM analysis

No.	Enzyme name	Substrate
1	Control	
2	Alkaline phosphatase	2-naphthyl phosphate
3	Esterase (C4)	2-naphtyl butyrate
4	Esterase Lipase (C8)	2-naphthyl caprylate
5	Lipase (C14)	2-naphthyl myristate
6	Leucine arylamidase	L-leucyl-2-naphthylamide
7	Valine arylamidase	L-valyl-2-naphthylamide
8	Cystine arylamidase	L-cystyl-naphthylamide
9	Trypsin	*N*-benzoyl-D-L-arginine-2-naphthylamide
10	α-chymotrypsin	*N*-glutaryl-phenylalanine-2-naphtylamide
11	Acid phosphatase	2-naphthyl phosphate
12	Naphthol-AS-BI-	Naphthol-AS-BI-phosphate

	phosphohydrolase	
13	α-galactosidase	6-Br-2-naphthyl-α-D-galactopyranoside
14	β-galactosidase	2-naphthyl-ß-D-galactopyranoside
15	β-glucuronidase	Naphthol-AS-BI-ßD-glucuronide
16	α-glucosidase	2-naphthyl-α-D-galactopyranoside
17	β-glucosidase	6-Br-2-naphthyl-ß-D-glucopyranoside
18	N-acetyl-β-glucosaminidase	1-naphthyl-N-acetyl-ß-D-glucosaminide
19	α-mannosidase	6-Br-2-naphthyl-α-D-mannopyranoside
20	α-fucosidase	2-naphthyl-α-L-fucopyranoside

3.5.3 Assessment of biofilm formation of *C. glabrata*

The method for biofilm formation was adapted from a procedure described by Jin *et al.* (2004). Yeast isolates were cultured on SDA at 30ºC for 24 hours. The yeast were suspended in 2 ml RPMI 1640 medium and adjusted to a final concentration of approximately 1 x 10^7 cells/ml using a spectrophotometer (OD_{530} = 0.38). Biofilms were produced on sterilized, polystyrene, flattened-bottom 96-wells microtiter plates (Nunclon, USA). For attachment phase, 100 µl of the adjusted cell suspension was transferred to each well. No cells were added to the final column of each microtiter plate to serve as negative control. The plate was incubated at 37ºC for 1.5 hours with constant shaking at 75 rpm. Following the attachment phase, unattached cells were removed. The wells were washed with PBS. Fresh RPMI 1640 medium was then added into each well. The plate was incubated at 37ºC for 24 to 72 hours with constant shaking at 75 rpm to allow biofilm growth.

3.5.4 Biofilm quantitation by tetrazolium salt [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[9phenylamino) carbonyl]-2H-tetrazolium hydroxide] (XTT) reduction assay

Biofilm metabolic activity was measured by the XTT reduction assay. Briefly, XTT (Sigma-Aldrich, USA) solution (1 mg/ml in PBS) was filter-sterilized through a 0.22 µm-pore-size filter and stored at -80ºC. For menadione (Sigma-Aldrich, USA) solution (0.4 mM) was prepared and filter-sterilized immediately before each assay. Prior to each assay, XTT solution was thawed and mixed with the menadione solution at a ratio of 5 to 1 by volume. After biofilm formation, the wells were washed three times with 200 µl PBS (Ramage *et al*., 2001). A total of 200 µl of PBS and 12 µl of XTT-menadione solution were added to each well (Appendix A). The microtiter plate was then incubated in the dark for 1 h at 35ºC. After incubation, 100 µl of the reaction solution was transferred to a new rounded-bottom microtiter plate and the absorbance was measured using spectrophotometer plate at 490 nm. The experiment was performed with eight replicates for each strain. The absorbance values of negative control wells (containing no cells) were subtracted from the test wells.

CHAPTER 4

RESULTS

4.1 Growth reactions of *C. glabrata* isolates on Chromagar medium

Table 4.1 shows the growth morphology of *C. glabrata* isolates on Chromagar medium after 48 hours of incubation. Of the 185 isolates tested, 129 (70 %) *C. glabrata* isolates produced purple colony, whereas 56 (30 %) of them produced white or creamy colonies. The growth morphology of three representative isolates *C. glabrata* and *C. nivariensis* is shown in Figure 4.1.

Table 4.1: Growth morphology of C. *glabrata* isolates on Chromagar medium.

Specimen type	**No. (%) isolates**	**No. (%) isolates with purple colonies**	**No. (%) isolates with white colonies**
Vaginal swab	142(76.8)	96(67.6)	46(32.4)
Blood	11(5.9)	9(80.0)	2(20.0)
Urine	14(7.6)	10(71.4)	4(28.6)
Respiratory secretions	4(2.2)	2(50.0)	2(50.0)
Others	14(7.6)	12(85.7)	2(14.3)
Total	185(100)	129(70.0)	56(30.0)

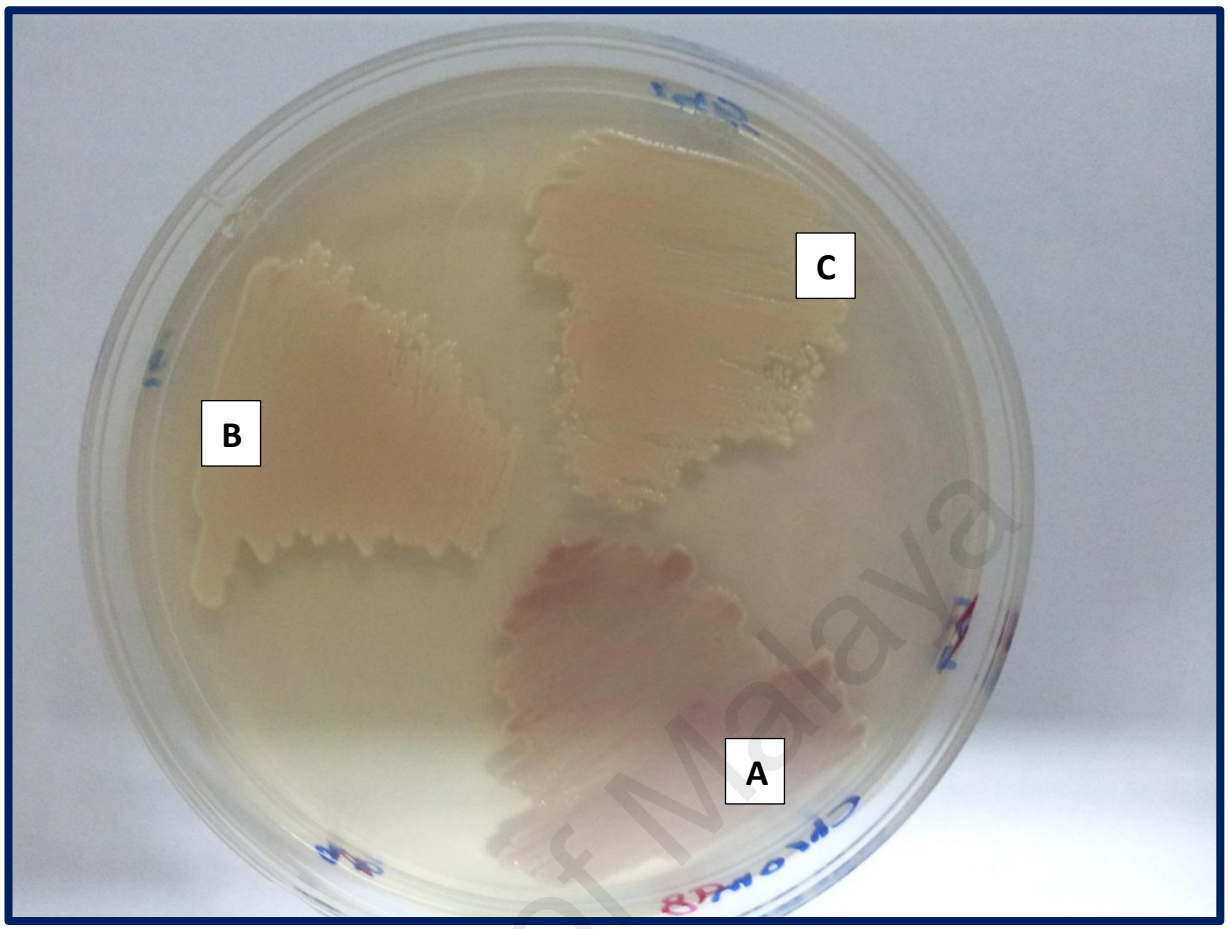


Figure 4.1: Colony colour of C. *glabrata* and *C. nivariensis* on a CHROMagar plate. (A) *C. glabrata,* (B & C) *C. nivariensis.*

4.2 Molecular screening of *C. nivariensis* and *C. bracarensis*

Figure 4.2 shows the results of the Singleplex PCR assay for some representative isolates investigated in this study. Four isolates (2.2 %) (designated as Cn139, Cn145, Cn160 and Cn189) were identified as *C. nivariensis* based on the observation of 665 bp DNA fragments on the agarose gel. While Cn139 was an isolate from a patient with bloodstream infection, Cn145, Cn160 and Cn189 were the serial isolates obtained from a patient with vaginitis. The remaining 181 isolates generated PCR products of 1,061 bp and were thus identified as *C. glabrata*. No *C. bracarensis* was identified in this study.

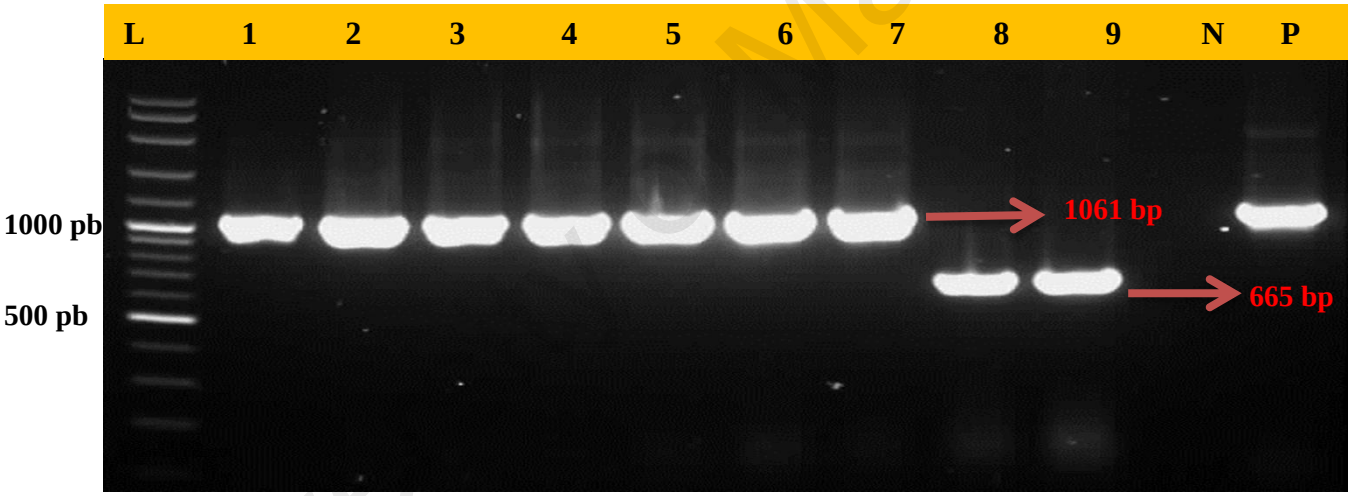


Figure 4.2 PCR Amplification of *RPL31*, a gene encoding for a protein component of the large (60S) ribosomal subunit for discrimination of *C. glabrata* (CG), *C. nivariensis* (CN) and *C. bracarensis*.

Lane: L (100bp DNA marker); 1(Cg158), 2(Cg159), 3(Cg161), 4(Cg162), 5(Cg163), 6(Cg164),7(Cg165) ,8 (Cn145), 9 (Cn139), N (Negative Control=distilled water), P (Positive Control=*C. glabrata* isolate , Cg 10)

4.3 Phenotypic and molecular identification of *C. glabrata*

The results of API 20 C AUX tests for 3 randomly selected *C. glabrata* and 3 *C. nivariensis* isolates identified in this study are shown in Table 4.2. Figure 4.3 is a diagram of the API test kit. All three *C. glabrata* isolates showed identical profile codes (2000040) which matched to that of *C. glabrata* in the database. Two *C. nivariensis* isolates (Cn139 and Cn145) had the profile codes (6000044) which matched 45.80% and 47.95% to *C. norvegensis* in the database, while the other isolate (Cn160) had the profile code of 6000040 which matched 42.90% to *C. glabrata*. Glucose assimilation was the only positive reaction noted for the *C. nivariensis* isolates after 48 hours of incubation.

Table 4.2: Results of PCR identification, API profiles, trehalose test and Chromagar growth reactions of *C. glabrata* and *C. nivariensis* isolates

Isolate label	PCR Identification	API code	Yeast identity, percentage accuracy	Result of trehalose assimilation test (API test)	Colony colour on Chromagar Medium
Cg159	*C. glabrata*	2000040	*C. glabrata*, 99.3%	Positive	Purple
Cg 157	*C. glabrata*	2000040	*C. glabrata*, 99.0%	Positive	Purple
Cg152	*C. glabrata*	2000040	*C. glabrata*, 99.3%	Positive	Purple
Cn139	*C. nivariensis*	6000044	*C. norvegensis*, 47.95%	Positive	White
Cn145	*C. nivariensis*	6000044	*C. norvegensis*, 45.80%	Positive	White
Cn160	*C. nivariensis*	2000040	*C. glabrata*, 42.90%	Positive	White

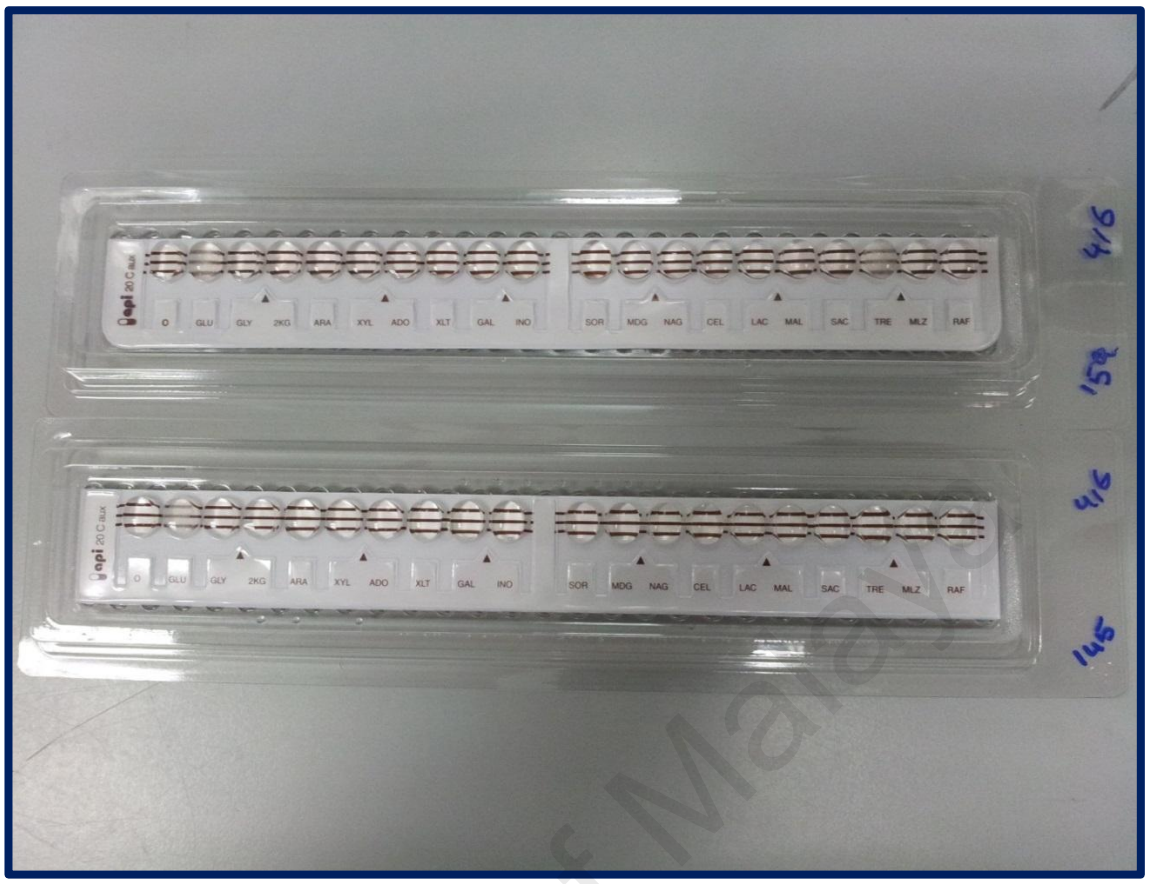


Figure 4.3: Result of *C. glabrata* API 20 C AUX test after 48h of incubation at 37°C.

C. glabrata Cg 159: *C. glabrata*, Glucose and trehalose positive

C. nivariensis Cn 145: *C. nivariensis*, Glucose positive, trehalose negative

4.4 Phenotypic and molecular identification of *C. nivariensis*

The origin and details of the *C. nivariensis* isolates identified in this study are shown in Table 4.3. As *C .nivariensis* was identified for the first time in Malaysia, the isolates were subjected to further testing using phenotypic and molecular methods.

Both *C. nivariensis* isolates (Cn139, and Cn145, as a representative isolate from each patient) grew as white, round, convex and smooth colonies on the potato dextrose agar and chromogenic agar (Table 4.3). The yeast cells were ovoid to subspherical on cornmeal agar. Short pseudohyphae were observed on the cornmeal agar for some yeast cells for Cn145 strain after incubation (Figure 4.5). Cn139 produced positive result using Glabrata-Quick™ kit, but not Cn145. The results of Glabrata Quick Kit test for *C. nivariensis strain*, Cn139 are shown in Figure 4.4.

Table 4.3: Phenotypic characteristic and antifungal susceptibility profiles of *C. nivariensis* isolates

Isolate	Source	CHROMagar reaction	Trehalose fermentation (Glabrata Quick kit)	Pseudohypha
Cn139	Blood	White	Positive	No
Cn 145	Vagina	White	Negative	Yes

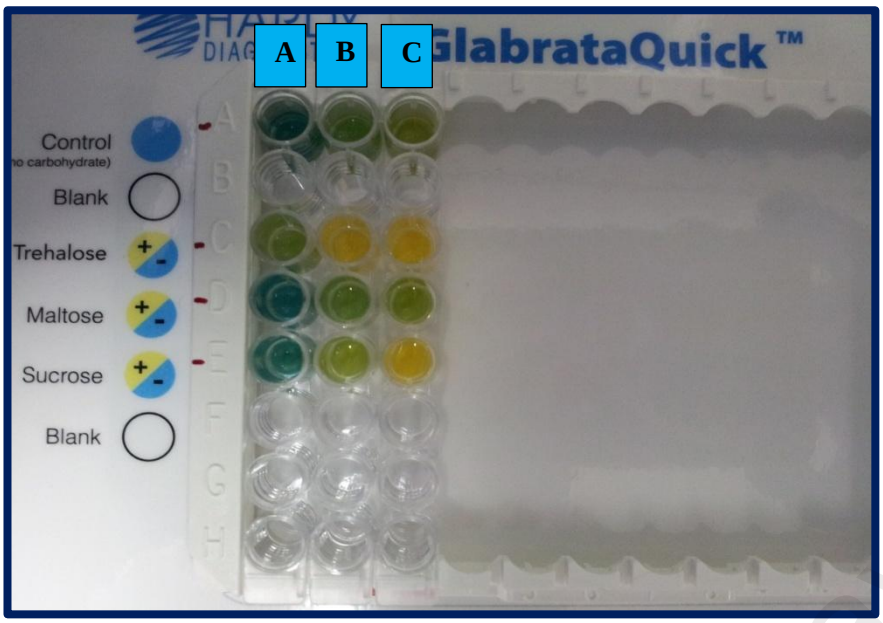


Figure 4.4: The reaction of Glabrata Quick test after 2 h of incubation. A colour change from blue or blue-green to yellow or greenish-yellow was an indication of positive reaction.

A) *C. nivariensis* isolated from a blood sample (UMMC 139): Trehalose (borderline), Maltose (negative), Sucrose (negative)

B) *C. glabrata* (negative control): Trehalose (Positive), Maltose (negative), Sucrose (negative)

C) *C. guilliermondii* (negative control): Trehalose (Positive), Maltose (negative), Sucrose (Positive)

Growth of *C. nivariensis* on Cornmeal agar

Short pseudohyphae were observed on the cornmeal agar for Cn145 strain after incubation (Figure 4.5). No hypha was observed for Cn139.

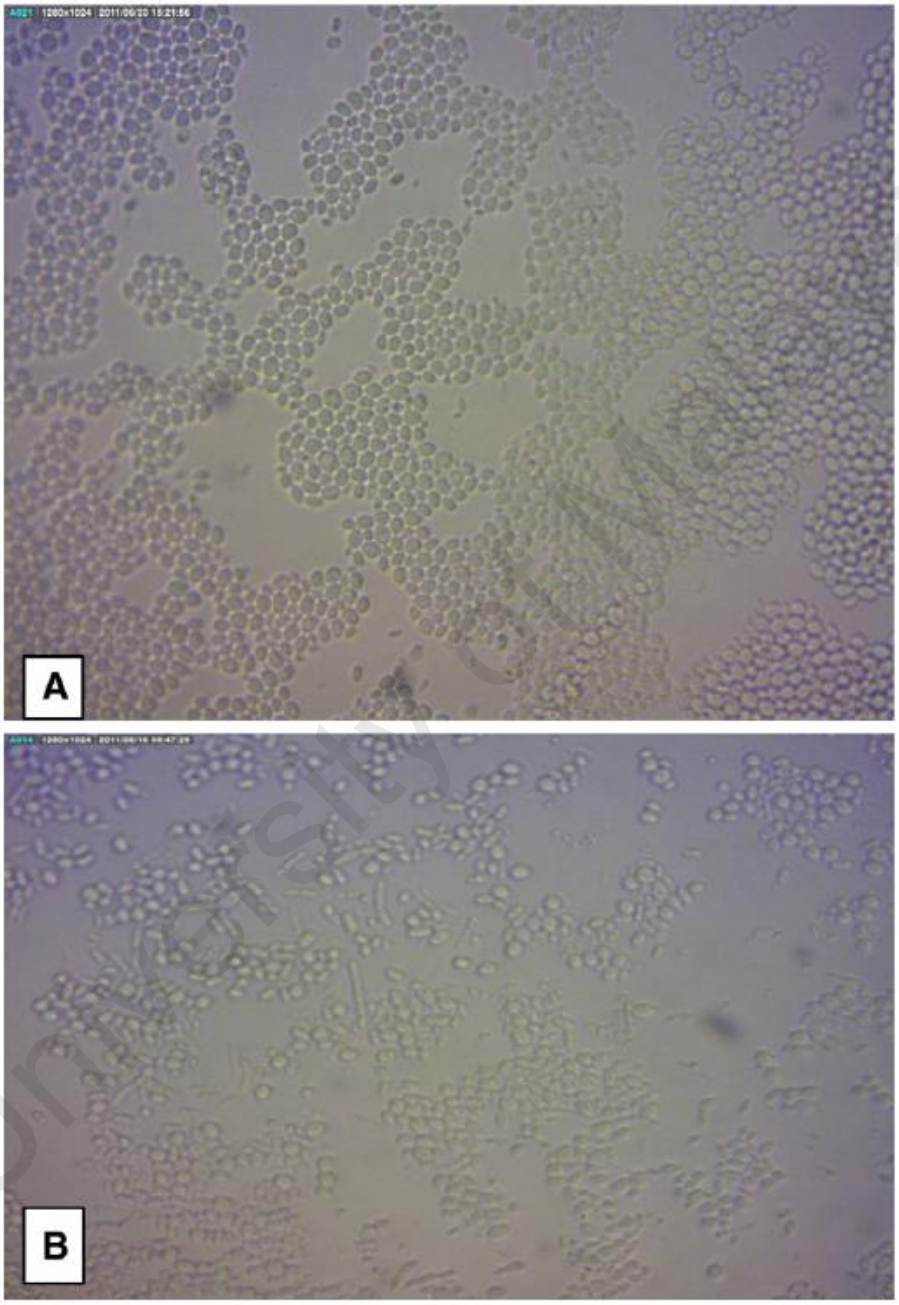


Figure 4.5: Growth morphology of *C. nivariensis* on cornmeal agar with Tween 80 after incubation at 30°C for 72 h. A, Cn139; B, Cn145

4.5 Molecular identification of *C. nivariensis*

The sequences of the ITS region (656 nucleotides, Genbank accession KF874486 and KF874487) and D1D2 domain (519 nucleotides, Genbank accession no. KF874488 and KF874489) of two *C. nivariensis* isolates (Cn139 and Cn145) were determined and deposited in the GenBank database. The sequences of the isolates are identical (100% matching) to those of *C. nivariensis* CBS 9983-type strain (Table 4.4).

Table 4.4: The sequencing result of ITS and D1 D2 regions for two *C. nivariensis* isolates

***C. nivariensis* Isolate**	**Source**	**GenBank submission (bp)**	
		ITS region	**D1D2 domain**
Cn139	Blood culture	KF874486 (656)	KF874488 (519)
Cn145	High vaginal swab	KF874487 (656)	KF874489(519)

4.6 Amplification and sequence analysis of the internal transcribed spacer (ITS) region of *C. glabrata* isolates

4.6.1 Sequence determination and analysis of *C. glabrata*

The amplified products of *C. glabrata* and *C. nivariensis* were analysed using agarose gel electrophoresis and the image for some of the representative isolates is shown in Figure 4.6. All isolates produced amplified fragments of about 800 bp**.** Based on the ITS sequences (ranging from 619 to 624 bp) obtained from 35 randomly selected *C. glabrata* isolates, a total of 9 ITS sequence types (G1-G9) were differentiated. The distribution of the isolates (majority were vaginal isolates) according to their respective sequence types is shown in Table 4.5.

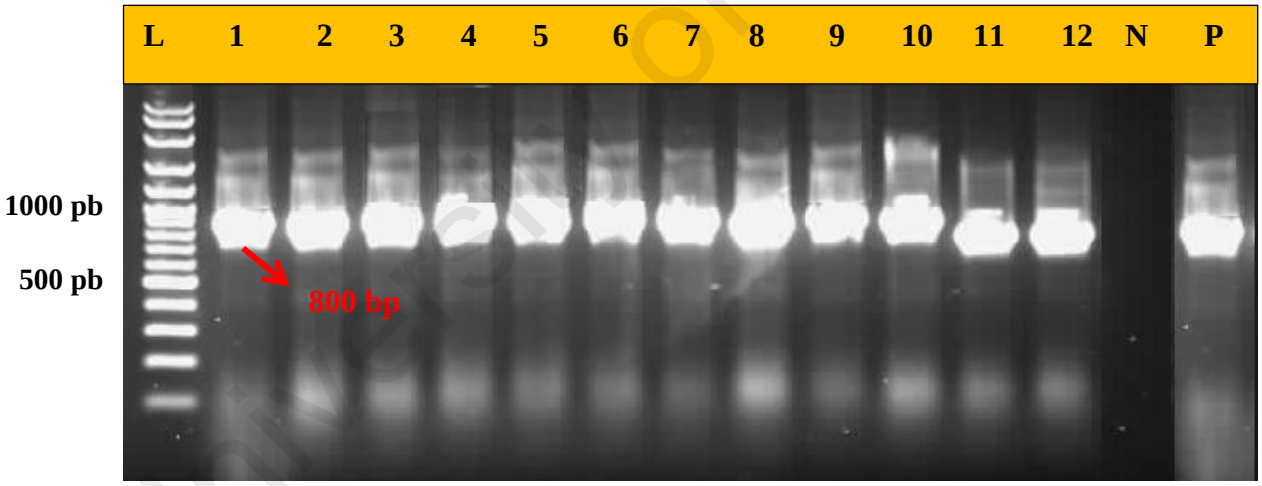


Figure 4.6. Agarose gel electrophoretic analysis of the amplified products (ITS gene region) of some representative *C. glabrata* and *C. nivariensis* isolates. All produced amplified fragments of about 800 bp. L = molecular weight ladder (100bp).

1, Cg 11; , 2, Cg 19; 3, Cg 24; 4, Cg 30; 5, Cg 53; 6, Cg 55; 7, Cg 56; 8, Cg106; 9, Cg 108; 10, Cn 139; 11, Cn 145; Cg = *C. glabrata;* Cn= *C. nivariensis;* P, positive control (a laboratory strain of *C. glabrata,* Cg10), N, negative control (distilled water)

Table 4.5: Source of *C. glabrata* isolates subjected to amplification and sequence analysis of ITS gene region.

No.	Isolate No. (Lab no.)	Specimen	Chromagar growth reaction	ITS sequence type
1	Cg120(1172)	HVS	White	G1
2	Cg131(1079)	BAL	White	G1
3	Cg25(9655)	HVS	Purple	G1
4	Cg31(2558)	HVS	Purple	G1
5	Cg136(0116)	HVS	White	G1
6	Cg141(6138)	HVS	White	G1
7	Cg152(8798)	Urine	Purple/White	G1
8	Cg186(4585)	HVS	Purple	G1
9	Cg195(7085)	Urine	Purple	G1
10	Cg198(7825)	HVS	Purple	G1
11	Cg197(7705)	HVS	White/Purple	G2
12	Cg199(0289)	Urine	White	G2
13	Cg19(7275)	HVS	Purple	G3
14	Cg21(6340)	HVS	Purple	G3
15	Cg185(4914)	HVS	Purple	G3
16	Cg135(0365)	HVS	White	G4
17	Cg138(0940)	HVS	White	G4
18	Cg162(7185)	HVS	Purple	G4
19	Cg181(5114)	Sputum/F	Purple	G4
20	Cg183(2564)	Urine	Purple	G4
21	Cg132(9537)	HVS	White	G5
22	Cg157(1802)	HVS	White	G5
23	Cg182(2710)	Urine	Purple	G5

24	Cg32(2960)	HVS	Purple	G6
25	Cg76(1670)	B/C	Purple	G6
26	Cg84(7103)	E/F	Purple	G6
27	Cg89(7103)	HVS	Purple	G6
28	Cg119(0339)	P/F	White	G6
29	Cg200(1176)	HVS	Purple	G6
30	Cg150(5523)	HVS	White/Purple	G7
31	Cg158(5959)	Urine	Purple	G7
32	Cg172(5927)	HVS	Purple	G7
33	Cg173(8188)	Sputum/F	Purple	G7
34	Cg163(5206)	HVS	White	G8
35	Cg194(5163)	HVS	Purple	G9

Peritoneal fluid (P/F), Endocervical swab (E/S), High vaginal swab (HVS), Blood Culture (B/C), Bronchoalveolar lavage (BAL)

The ITS sequences for the isolates matched with at least one of the *C. glabrata* isolates in the GenBank database. The three most predominant ITS sequence types were ITS sequence type 1, 6 and 4. (Table 4.6) The sequence similarity of *C. glabrata* isolates ranged from 98.7 to 100% when compared to the reference strain, CBS 138 (Table 4.7). The unrooted dendrogram (Figure 4.7) constructed based on the partial ITS1 gene sequences of *C. glabrata* isolates shows the clustering of the isolates with their corresponding reference sequences. The ITS sequences are shown in Figure 4.8.

Table 4.6: Distribution of 35 *C. glabrata* isolates according to their respective ITS sequence type.

ITS sequence type	No (%) isolates	Matching strain, GenBank accession no.	% matching	Specimen, geographical location (reference)
1	10(28.5 %)	CBS 12440, KJ009319	100	Knee arthritis, Iran (Erami *et al*., 2014)
2	2(5.7 %)	ZB066, FJ697172	100	Stomach mucous membrane, China (Gong *et al*., 2012)
3	3(8.5 %)	CBS 138, AY198398	100	Blood, Ireland (Wong *et al*., 2003)
4	5(14.2 %)	ATCC 90030, AY939793	100	Clinical sample, Germany (Leinberger *et al*., 2005)
5	3(8.5 %)	W56873, GU199447	100	Blood, Denmark (Mirhendi *et a*l., 2011)
6	6(17.6 %)	CNRMA7.137, KP131704	100	Clinical sample, Australia (Irinyi *et al*., 2015)
7	4(11.4 %)	CNRMA 11.192, KP131702	100	Clinical sample, Australia (Irinyi *et al*., 2015)
8	1(2.8 %)	CNRMA 6.53, KP131703	100	Clinical sample, Australia (Irinyi *et al*., 2015)
9	1(2.8 %)	H371B, KP675134	100	Oral region, China (Gong *et al*., 2015)

Table 4.7: Sequence similarity of *C. glabrata* isolates, according to ITS sequence type.

Strain	Strain CBS 138 (AY198398)	Strain CBS 12440 (KJ009319)	Strain ZB066 (FJ697172)	Strain ATCC 90030 (AY939793)	Strain W56873 (GU199447)	Strain CNRMA7.1370 (KP131704)	Strain CNRMA11.192 (KP131702)	Strain CNRMA6.53 (KP131703)	Strain H371B (KP675134)
Strain CBS 138 (AY198398)-G3	100.0								
Strain CBS 12440 (KJ009319)-G1	99.0	100.0							
Strain ZB066 (FJ697172)-G2	99.3	99.6	100.0						
Strain ATCC 90030 (AY939793)-G4	99.5	99.5	99.5	100.0					
Strain W56873 (GU199447)-G5	98.7	98.7	98.7	99.1	100.0				
Strain CNRMA7.1370 (KP131704)-G6	99.3	99.3	99.3	99.8	99.0	100.0			
Strain CNRMA11.192 (KP131702)-G7	99.1	98.8	99.1	99.3	98.8	99.5	100.0		
Strain CNRMA6.53 (KP131703)-G8	99.3	99.3	99.3	99.8	99.0	99.6	99.5	100.0	
Strain H371B (KP675134)-G9	99.3	99.3	99.6	99.5	98.7	99.3	99.5	99.6	100.0

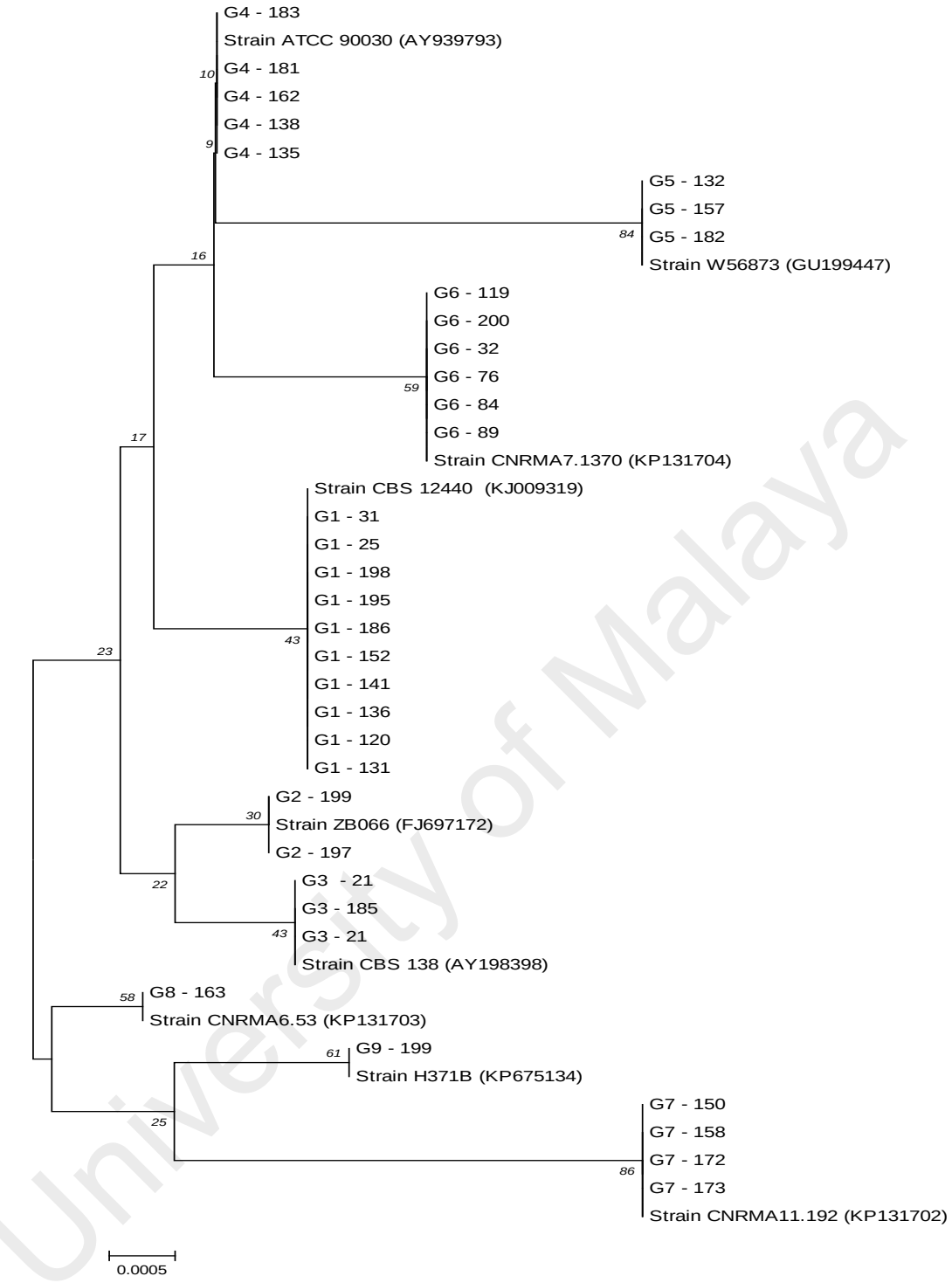


Figure 4.7 Dendrogram constructed based on the ITS sequences of *C. glabrata* isolates. The details of *C. glabrata* included in the dendrogram are shown in Table 4.5. Bootstrap values are indicated at the nodes.

Figure 4.8 Sequence determination and analysis of *C. glabrata isolates* based on ITS gene region. (Grafic fasta format in Appendix C)

4.7 Sequence analysis of the ITS1 gene region of *C. nivariensis*

Figure 4.9 is a dendrogram which was constructed when the entire ITS gene sequences (656 bp) of the *C. nivariensis* isolates (Cn139 and Cn145) were aligned with those of *C. glabrata*. In the dendrogram, two main clusters were distinguished, with one includes all *C. glabrata* isolates and the other one, mainly of *C. nivariensis*, with 100% bootstrap support.

Following the published study of Sharma *et al.* (2013), the partial ITS1 sequences (293 nucleotides) were extracted from the ITS gene region of *C. nivariensis* for comparison with those available in the GenBank database. Table 4.8 shows the origin and details of the Malaysian *C. nivariensis* isolates and other *C. nivariensis* reported from other regions of the world. There were high sequence similarities (ranging from 97.6 to 100 %, 0–7 nucleotide differences) between the two *C. nivariensis* isolates in this study with others in the GenBank database. Figure 4.10 demonstrates the clustering of the Malaysian isolates with those from United Kingdom (NCPF 8842–8853 and C.niv9); Spain (CBS 9983-9985), USA (IDR110 0004443, IDR1100004444, IDR1100006818), India (VPCI 826/P/12 and S-32) and Indonesia (CBS 10161). Three vaginal isolates from India (VPCI1028/12, VPCI818/P/12 and VPCI774/P/12), two isolates from China (FC531 and PUMY019), H41019 and the United Kingdom isolate (URCn2) were grouped on different branches within the *C. nivariensis* cluster.

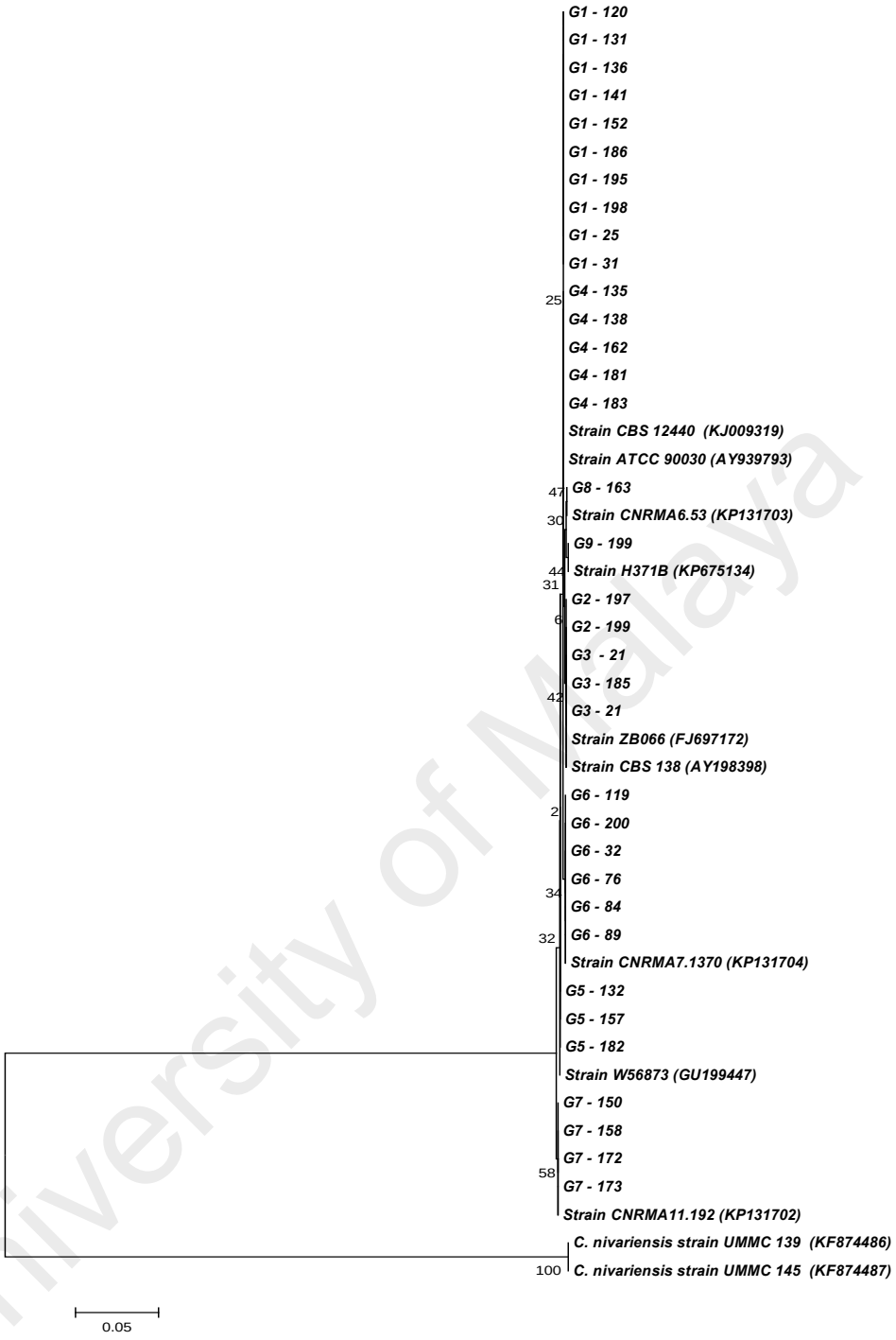


Figure 4.9: Sequence analysis of the ITS gene region of *C. glabrata* and *C. nivariensis* investigated in this study. The details of *C. glabrata* included in the dendrogram are shown in Table 4.8. Bootstrap values are indicated at the nodes.

Table 4.8: *C. nivariensis* strains included for partial ITS1 sequence analysis in this study.

Isolate label	Isolation site	Clinical details	Country	GenBank accession number	References
CBS 9983	Blood culture	Biliary pancreatitis, functional renal failure	Spain	GU199443	Alcoba-Florez *et al.*, 2005
CBS9984	Bronchoalveolar lavage fluid	Multiple pulmonary abscesses	Spain	AY620959	Alcoba-Florez *et al.*, 2005
CBS 9985	Urine	Lumbar pain and mild dyspnea after myomectomy	Spain	GU199444	Alcoba-Florez *et al.*, 2005
8842	Mouth	Oral candidiasis	UK	AM745269	Borman *et al.*, 2008
8843	Pelvic collection	Not stated	UK	AM745271	Borman *et al.*, 2008
8844	Blood culture	Neutropenia, AML	UK	AM745273	Borman *et al.*, 2008
8845	Mouth	Oral candidosis	UK	AM745275	Borman *et al.*, 2008
8846	Not stated	Pneumonia	UK	AM745277	Borman *et al.*, 2008
8847	Ascitic fluid	Malignancy	UK	AM745279	Borman *et al.*, 2008
C.niv9	Not stated	Pneumonia	UK	AM745281	Borman *et al.*, 2008
8848	Mouth	Oral candidosis, neutropenia	UK	AM745283	Borman *et al.*, 2008
8849	Exit site swab	CAPD	UK	AM745285	Borman *et al.*, 2008
8850	Peritoneal fluid	Peritonitis	UK	AM745287	Borman *et al.*, 2008
8851	Lung biopsy	Not stated	UK	AM745289	Borman *et al.*, 2008
8852	Blood culture	Not stated	UK	AM745291	Borman *et al.*, 2008
8853	Not stated	Pneumonia	UK	AM745293	Borman *et al.*, 2008
CBS 10161	Oral rinse	HIV	Indonesia	GU199441	Wahyuningsih *et al.*, 2008
S32	Sputum	HIV	India	FM955316	Chowdhary *et al.*, 2010
VPCI 1293/08	Blood culture	Diabetes	India	FM955317	Chowdhary *et al.*, , 2010
VPCI1028/12	Bronchoalveolar lavage fluid	Lung Carcinoma	India	KC479178	Sharma *et al.*, 2013
VPCI818/P/12	Vagina swab	Vaginitis	India	KC479176	Sharma *et al.*, 2013
VPCI774/P/12	Vaginal swab	Vaginitis	India	KC479174	Sharma *et al.*, 2013
H41019	Not stated	Not stated	AdvanDx	GU199442	Mirhendi *et al.*, 2011
UMMC 139	Blood culture	Prolonged hospital stay, use of TPN	Malaysia	KF874486	This study
UMMC 145	High Vaginal swab	Vaginitis	Malaysia	KF874487	This study
IDR1100006818, IDR1100004444, IDR1100004443	Not stated	Not stated	USA	JN675320, JN675319, JN675318	Chaturvedi & Charturvedi, unpublished
FC531	Urine	Not stated	China	KF410863	Cao *et al.*, unpublished
PUMY019	Vagina swab	Not stated	China	JN391289	Zhao *et al.*, unpublished
URCn2	Urine	Not stated	UK	JN657499	Jones *et al.*, unpublished

NA, not analyzed; AML, acute myeloid leukemia; CAPD, continuous ambulatory peritoneal dialysis; HIV, human immunodeficiency virus, TPN, total parenteral nutrition; UK, United Kingdom; USA, United States of America

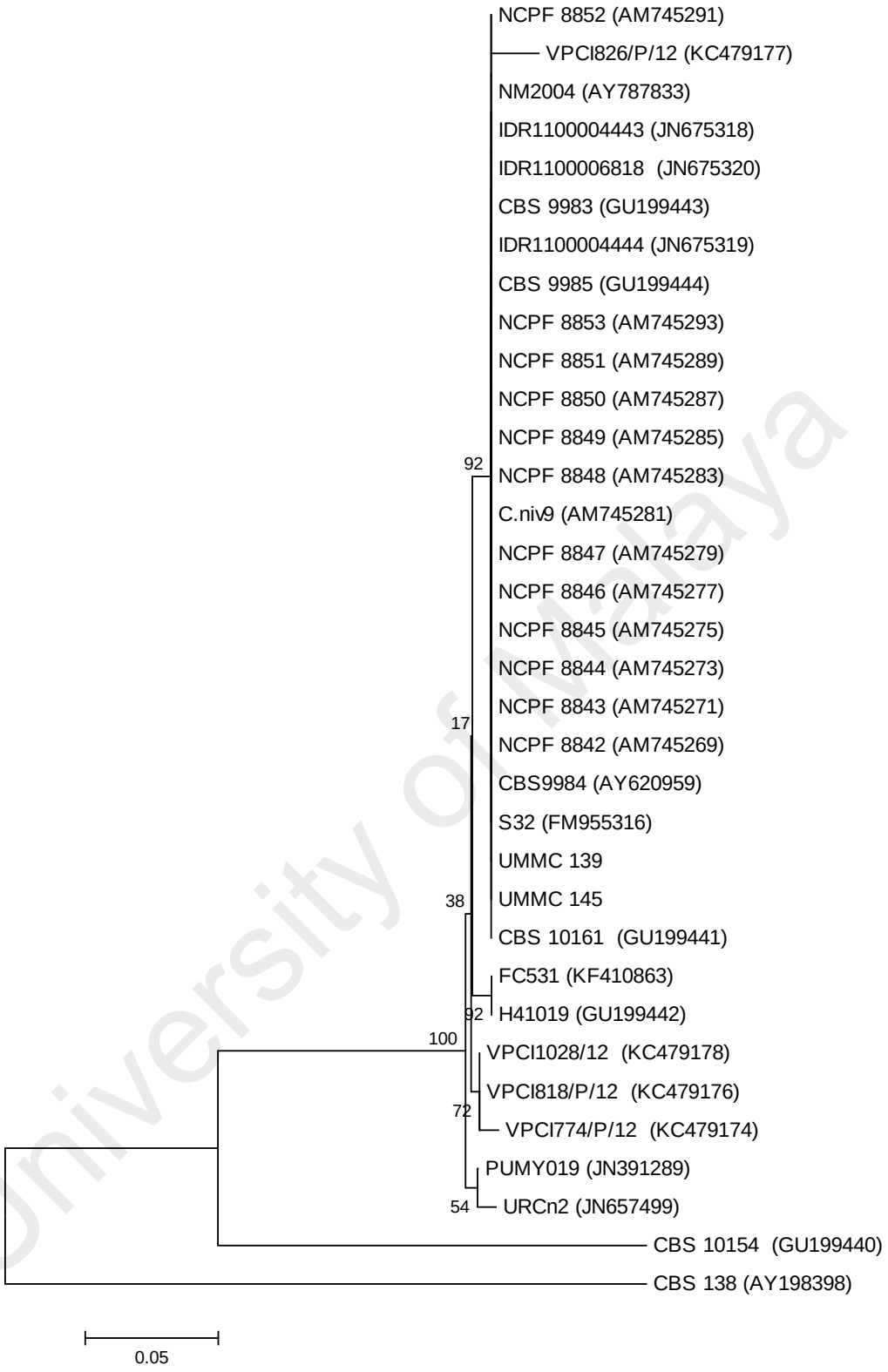


Fig 4.10 Dendrogram was constructed based on the partial ITS1 gene sequences of *C. nivariensis, C. bracarensis* CBS10154 and *C. glabrata* CBS138. The details of C. *nivariensis* included in the dendrogram are shown in Table 4.8. Bootstrap values are indicated at the nodes.

4.8 RAPD results for *C. glabrata* and *C. nivariensis*

RAPD analysis was performed for 58 isolates of which 16 were from blood cultures (5 isolates were added from an existing culture collection), 34 were from vaginal swabs, and 4 other samples from different sources (2 urine isolates, 2 sputum isolates) (Table 4.9). Four *C. nivariensis* isolates were also included in the investigation.

Primer M13 generated DNA fragments with higher intensity (as shown in Figure 4.11), as compared to those produced by the primer $(GTG)_5$ (Figure 4.12). However, more DNA fragments were observed when primer $(GTG)_5$ was used, particularly for *C. nivariensis* isolates. Table 4.9 summarizes the results of RAPD analysis for *C. glabrata* and *C. nivariensis* isolates. A total of 7 and 6 RAPD clusters were generated using M13 and $(GTG)_5$ primers, respectively (Figure 4.11 and Figure 4.12). The results in this study show genetic heterogeneity of the 16 blood culture isolates, as supported by the differentiation of the isolates into clusters 1, 3 and 5 in M13 fingerprints, and clusters 1, 3 and 4 in $(GTG)_5$ fingerprints (Table 4.9). In contrast, the 34 vaginal isolates were distributed in six and five clusters, based on the M13 and $(GTG)_5$ fingerprints, respectively. By combining the results obtained from each dendrogram, a total of 15 RAPD types (A-O) were assigned to each *C. glabrata* isolate (Table 4.10). Five RAPD types (A, B, C, I, M) were identified among the blood isolates, 10 RAPD types (D, E, F, G, H, I, K, L, N, O) were identified for the vaginal isolates, and two RAPD type were found for urine and sputum isolates (B, I, J, H), respectively. RAPD type B was shared amongst one urine and three blood culture isolates. RAPD type H was shared amongst one isolate from sputum and three isolates from the vagina sites. RAPD type I was shared amongst isolates from one blood culture, three vaginal swabs and one urine culture. The RAPD analysis differentiated *C. nivariensis* isolates as a unique cluster in both dendrograms, demonstrating more than 60% difference from those *C. glabrata*

isolates (Figure 4.11 and 4.12). Cn139 exhibited different RAPD type (Q) fingerprint as compared to Cn145, Cn160 and Cn189 (P) (Table 4.10).

Table 4.9: RAPD analysis of 54 *C. glabrata* and 4 *C. nivariensis* isolates. Blood culture (BC): 16 isolates, high vaginal swab (HVS) isolates: 32 isolates, low vaginal swab (LVS) isolates: 2 isolates, Urine: 2 isolates, sputum: 2 isolates) with primer M13 and $(GTG)_5$ by RAPD techniques.

M13 primer (source of isolates)	**$(GTG)_5$ primer (source of isolates)**
Cluster 1 (15 isolates including 11 blood isolates, 2 HVS, 2 LVS isolates and 1 urine isolate)	Cluster 1 (8 isolates including 2 blood isolates, 5 HVS isolates and 1 sputum isolate)
Cluster 2 (including 4 HVS isolates)	Cluster 2 (including 1 HVS isolate, 3 blood isolates and1 urine isolate)
Cluster 3 (10 isolates including 6 HVS isolates, 1 blood isolates, 2 sputum isolates, and 1 urine isolate)	Cluster 3 (9 isolates including, 5 blood isolates, 1 HVS, 2 LVS isolates, and 1 urine isolate)
Cluster 4 (including 12 HVS isolates)	Cluster 4 (22 isolates including 16 HSV isolates, 6 blood isolates, and 1 sputum isolates)
Cluster 5 (including 4 blood isolates)	Cluster 5 (including 9 HVS isolates)
Cluster 6 (including 8 HVS isolates)	Cluster 6 (including 4 *C. nivariensis* isolates)
Cluster 7 (including 4 *C. nivariensis* isolates)	-

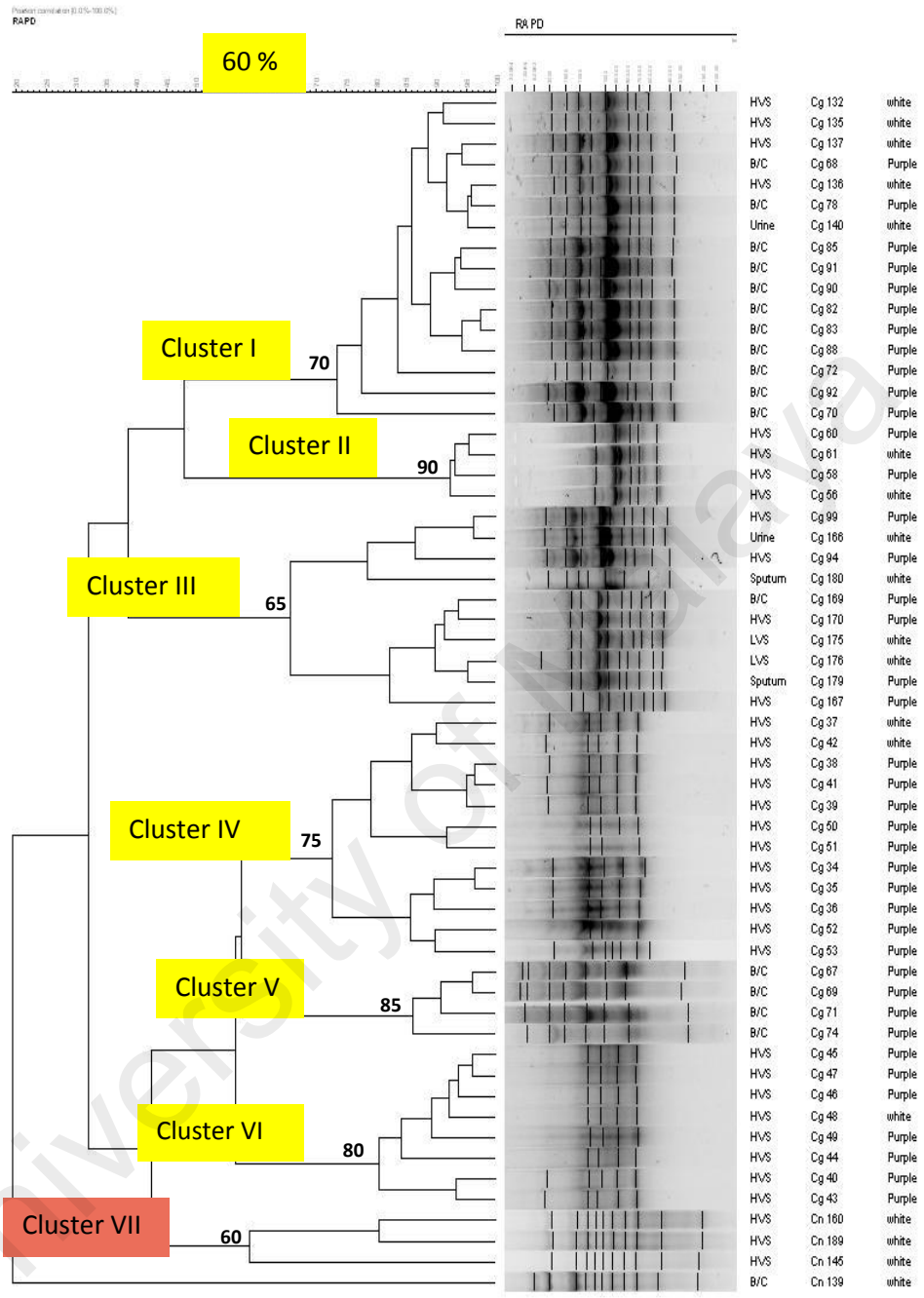


Figure 4.11: **RAPD fingerprinting patterns of 58 *C. glabrata* and *C. nivariensis* clinical isolates generated by primer M13. A total of 6 clusters were defined, when 60% similarity was used as a cut-off point, amongst the *C. glabrata* isolates (Castrillon *et al*., 2014) .HVS (High vaginal swab), B/C (Blood culture), LVS (Low vaginal swab)**

Note: Five blood culture isolates were added from an existing culture collection (2008-2009) (Appendix A).

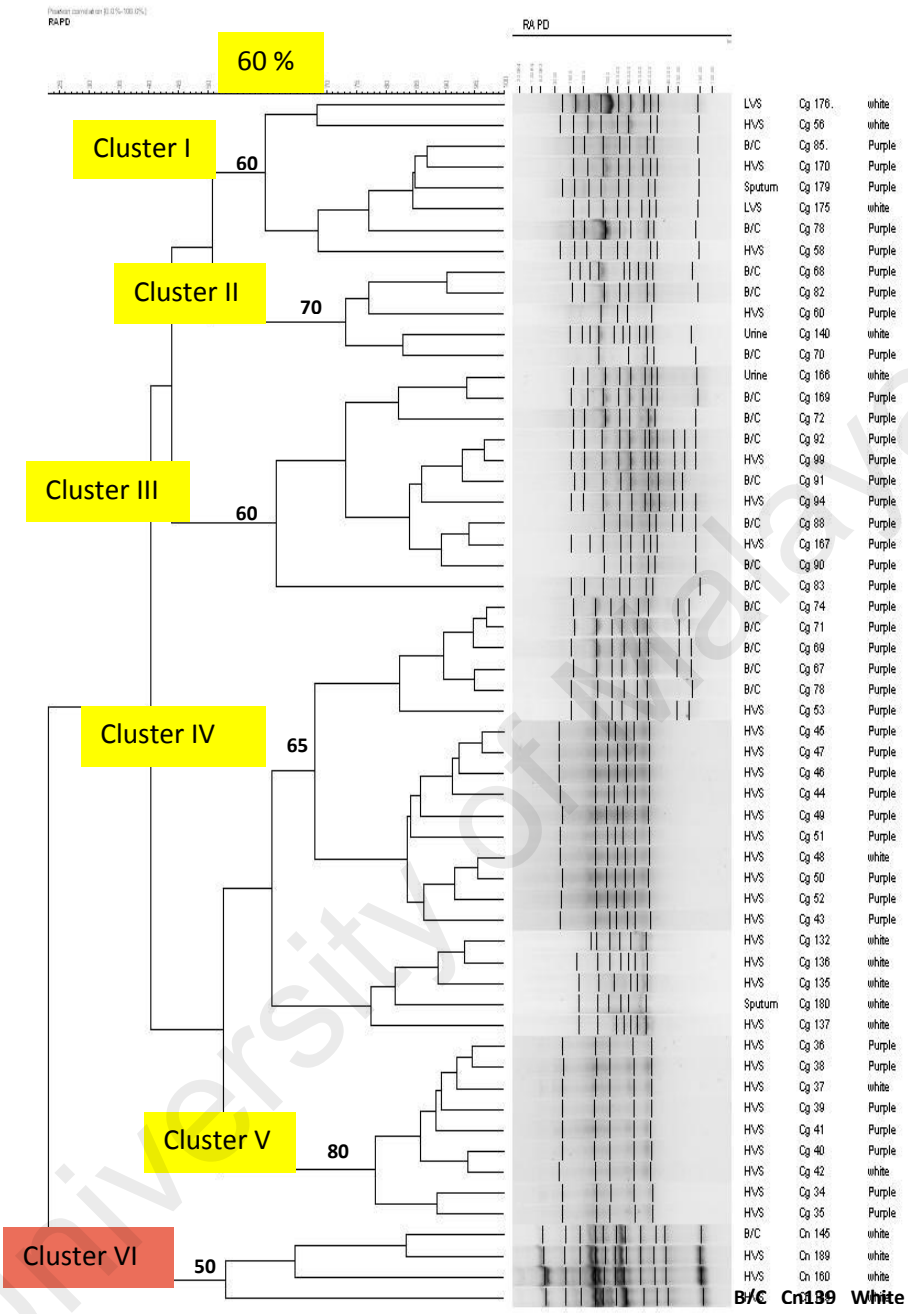


Figure 4.12: RAPD fingerprinting patterns of 58 *C. glabrata* and *C. nivariensis* clinical isolates generated by primer $(GTG)_5$. Five clusters were identified, when 60% similarity was used as a cut-off point, amongst the *C. glabrata* isolates (Castrillon *et al*., 2014). HVS (High vaginal swab), B/C (Blood culture), LVS (Low vaginal swab)

Note: Five blood culture isolates were added from an existing culture collection (2008-2009) (Appendix A).

Table 4.10. Genotyping of *C. glabrata* isolates based on M13 and $(GTG)_5$ dendrograms.

Strain no.	Source	M13 cluster	$(GTG)_5$ cluster	RAPD type	Clo MIC (µg/ml)	Biofilm Average OD readings	Phospholipase activity (Pz* value)
Cg 78	B/C	1	1	A	2	-	1
Cg 85	B/C	1	1	A	2	-	0.6
Cg 68	B/C	1	2	B	2	-	0.6
Cg 140	Urine	1	2	B	-	0.106±0.0219	1
Cg 82	B/C	1	2	B	0.5	-	0.4
Cg 70	B/C	1	2	B	0.1	-	0.45
Cg 91	B/C	1	3	C	0.5	-	0.6
Cg 90	B/C	1	3	C	4	-	0.5
Cg 83	B/C	1	3	C	0.3	-	0.4
Cg 88	B/C	1	3	C	4	0.229±0.0773	0.42
Cg 72	B/C	1	3	C	4	0.051±0.0105	0.64
Cg 92	B/C	1	3	C	2	0.201±0.1688	0.57
Cg 132	HVS	1	4	D	-	0.234±0.1057	0.4
Cg 135	HVS	1	4	D	-	-	0.5
Cg 137	HVS	1	4	D	-	0.240±0.1196	0.4
Cg 136	HVS	1	4	D	-	0.200±0.0720	0.45
Cg 58	HVS	2	1	E	0.5	-	0.51
Cg 56	HVS	2	1	E	0.25	-	0.53
Cg 60	HVS	2	2	F	1	-	0.57
Cg 61	HVS	2	4	G	2	-	0.6
Cg 170	HVS	3	1	H	-	-	0.65
Cg 175	LVS	3	1	H	-	0.134±0.1012	0.66
Cg 176	LVS	3	1	H	-	0.222±0.1145	0.56
Cg 179	Sputum	3	1	H	-	0.242±0.1996	0.48
Cg 99	HVS	3	3	I	1	0.104±0.0937	1
Cg 166	Urine	3	3	I	-	-	0.55
Cg 94	HVS	3	3	I	1	0.207±0.1007	0.53
Cg 169	B/C	3	3	I	-	0.297±0.1966	0.44
Cg 167	HVS	3	3	I	-	-	0.62
Cg 180	Sputum	3	4	J	-	0.204±0.1686	0.6
Cg 50	HVS	4	4	K	4	-	0.48
Cg 51	HVS	4	4	K	0.25	0.162±0.0321	0.5
Cg 52	HVS	4	4	K	0.125	0.193±0.0558	0.5
Cg 53	HVS	4	4	K	0.125	-	0.53
Cg 37	HVS	4	5	L	0.25	0.110±0.0015	0.56
Cg 42	HVS	4	5	L	0.5	-	0.5
Cg 38	HVS	4	5	L	0.125	0.115±0.0107	0.6
Cg 41	HVS	4	5	L	0.5	-	0.54
Cg 39	HVS	4	5	L	0.5	0.102±0.0220	0.6

Cg 34	HVS	4	5	L	0.25	0.115±0.0131	0.52
Cg 35	HVS	4	5	L	0.5	0.110±0.0177	0.57
Cg 36	HVS	4	5	L	0.5	0.095±0.0327	0.68
Cg 67	B/C	5	4	M	2	-	0.65
Cg 69	B/C	5	4	M	0.5	-	0.49
Cg 71	B/C	5	4	M	4	-	0.5
Cg 74	B/C	5	4	M	0.25	-	0.6
Cg 45	HVS	6	4	N	0.125	-	0.5
Cg 47	HVS	6	4	N	0.25	-	0.57
Cg 46	HVS	6	4	N	0.5	-	0.5
Cg 48	HVS	6	4	N	0.5	-	0.54
Cg 49	HVS	6	4	N	1	-	0.6
Cg 44	HVS	6	4	N	0.5	-	0.5
Cg43	HVS	6	4	N	0.25	-	0.5
Cg 40	HVS	6	5	O	0.25	-	0.52
Cn145	B/C	7	6	P	0.5	0.149±0.0206	0.54
Cn160	HVS	7	6	P	0.5	0.120±0.0007	0.61
Cn189	HVS	7	6	P	0.5	0.244±0.1162	0.5
Cn139	HVS	8	7	Q	0.125	0.238±0.1103	0.4

Cg, *C. glabrata;* Cn, *C. nivariensis;* HVS, high vaginal swab, B/C, blood culture; LVS, low vaginal swab; Clo, clotrimazole

4.9 Antifungal susceptibility testing

Figure 4.13 shows the antifungal susceptibility of *C. glabrata* isolates using E test. Based on the CLSI interpretation guide, none of the 80 *C. glabrata* and 4 *C. nivariensis* isolates tested were resistant against the four antifungal drugs. Table 4.11 shows the minimum inhibitory concentrations (MIC) of 80 *C. glabrata* isolates against amphotericin B (ranging from 0.004 – 1 µg/ml), fluconazole (ranging from 0.023 – 6 µg/ml), voriconazole (ranging from 0.002 - 0.047µg/ml) and caspofungin (ranging from 0.004 – 1.5 µg/ml), as determined by E-tests after 24h of incubation. The MICs for clotrimazole ranged from 0.03 - 8 µg/ml, A total of 51 (60%) isolates demonstrated resistance to clotrimazole (MICs ≥ 0.5 µg/ml). Of these resistant isolates, 25, 12, 9, 4, 1 isolates had MICs of 0.5, 1, 2, 4, 8 µg/ml, respectively. Most of the clotrimazole resistant isolates were distributed in the clusters 1and 4 in the M13 dendrogram (most isolates were from blood and vaginal swabs) and clusters 3 and 4 in the $(GTG)_5$ dendrogram.

The susceptibilities of the Malaysian *C. nivariensis* isolates against amphotericin B (ranging from 0.016–0.064 µg/ml), fluconazole (ranging from 0.75–1.5µg/ml), voriconazole (ranging from 0.023–0.032 µg/ml) and caspofungin (ranging from 0.028–0.047 µg/ml) are shown in Table 4.11. The MICs for clotrimazole for Cn139 and Cn 145 were 0.125 and 0.5 µg/ml, respectively. Hence, only one isolate (Cn145) was considered as clotrimazole resistant.

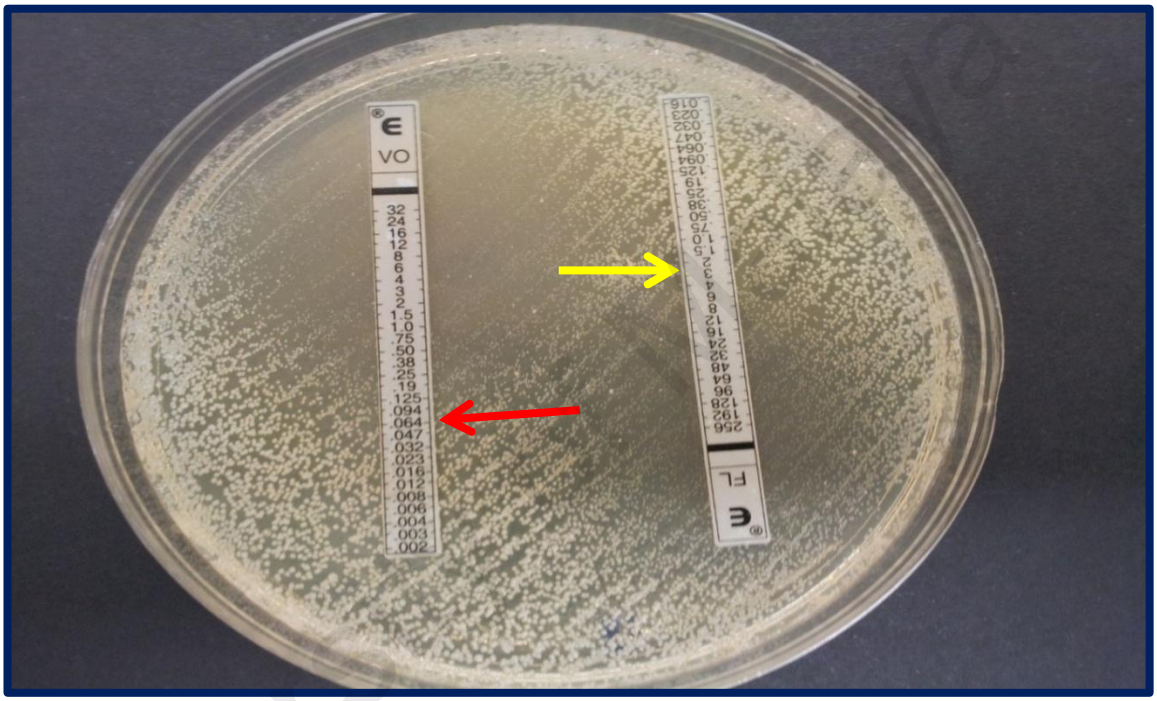


Figure 4.13: The antifungal susceptibility testing of *C. glabrata* using E test. The inhibition zone was read after 48 h of incubation.

FL (fluconazole) MIC: 3 µg/ml, VO (voriconazole) MIC: 0.064 µg/ml

Table 4.11: Antifungal susceptibility testing of *C. glabrata* and *C. nivariensis* isolates using E tests after 24 h of incubation

Candida isolates	Minimum inhibitory concentration (µg/ml)				
	Fluconazole	**Voriconazole**	**Clotrimazole**	**Amphotericin B**	**Caspofungin**
C. glabrata (n=80)					
MIC range	0.023 – 6	0.002 - 0.047	0.03 - 8	0.004 – 1	0.004 – 1.5
MIC_{50}	0.19	0.008	0.5	0.125	0.047
MIC_{90}	6	0.047	8	1	1.5
C. nivariensis (n=4)					
MIC range	0.75–1.5	0.023–0.032	0.125 – 0.5	0.016–0.064	0.028–0.047
MIC_{50}	0.25	0.008	0.5	0.016	0.023
MIC_{90}	0.5	0.008	0.5	0.032	0.032

4.10 Analyses of possible virulence factors of *C. glabrata* isolates

4.10.1 Phospholipase activity

In this study 97 *C. glabrata* isolates were included for testing of phospholipase activity (Table 4.12). *C. albicans* ATCC 90028, known as a phospholipase producer (Pz=0.48), was used as a positive control while *C. parapsilosis* ATCC 22019 (Pz=1.0) was used as a negative control. None of the 97 *C. glabrata* and 4 *C. nivariensis* produced the typical phospholipase activity (white precipitation zone surrounding yeast colonies). However, faint haziness zones were observed for 86 (89%) *C. glabrata* and four *C. nivariensis* isolates, reflecting the weak phospholipase activity of both *C. glabrata* and *C. nivariensis* isolates (Figure 4.14 & 4.15). The production of the weak phospholipase activity of *C. glabrata* isolates was quantitated as Pz*=a/b, whereby a= diameter of the colony (mm), b= diameter of the haziness zone (mm). Table 4.12 shows the Pz* values obtained from the isolates investigated in this study.

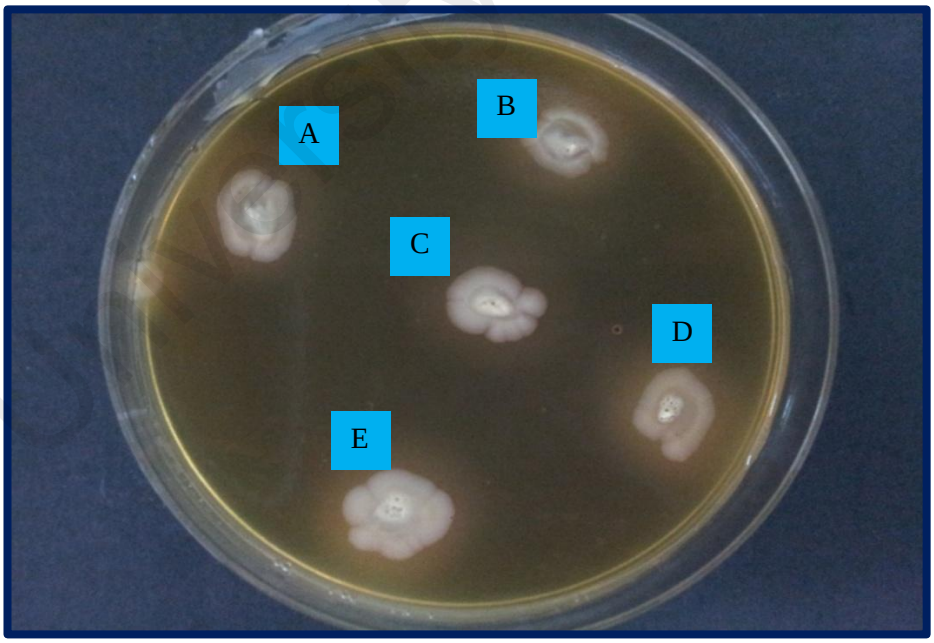


Figure 4.14: Egg-yolk agar plate inoculated with *C. glabrata* isolates incubated at 37°C for 7 days. Hazy area shows weak phospholipase activity.

A, Cg1(HVS); B, Cg2(HVS); C, Cg3(HVS); D, Cg4(HVS); E, Cg5(HVS)

Table 4.12: Phospholipase activity of *C. glabrata* and *C. nivariensis* isolates

***Candida* isolate**	**No. tested**	**No.(%) isolates producing white precipitation surrounding colony**	**Pz value (average)**	**No. (%) isolates producing haziness surrounding colony**	**Pz* value (average)**
C. glabrata	97	0(0)	1.0	86 (88.7%)	0.53 ±0.61
C. nivariensis	4	0(0)	1.0	4(100%)	0.51±0.75

Pz value of *C. albicans* ATCC 90028 = 0.480

Pz value of *C. parapsilosis* ATCC 22019 = 1.0

Note: The production of the phospholipase of the yeasts was denominated as Pz=a/b, as described by Price *et al* 1982.

Pz* value was used to indicate the weak phospholipase production of *C. glabrata* and C. *nivariensis*

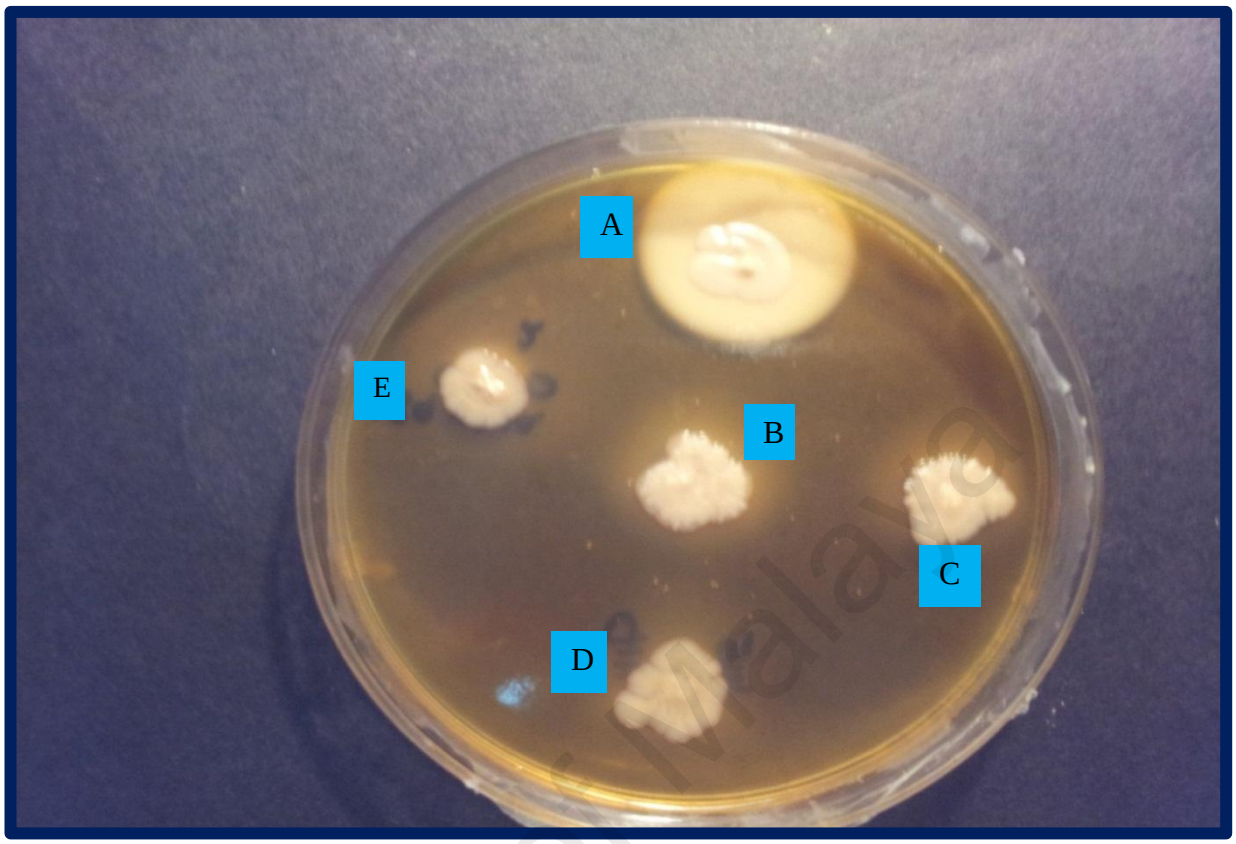


Figure 4.15: Egg-yolk agar plate inoculated with *C. nivariensis* isolates at 37C° for 7 days. Weak phospholipase activity was demonstrated by the hazy zone around *C. nivariensis* colony.

(A): *C. albicans* ATCC 90028, (B): *C. nivariensis* B 139(B/C),

(C): *C. nivariensis* 160 (HVS), (D): *C. nivariensis* 145 (HVS)

(E): *C. nivariensis* 189 (HVS)

4.10.2 Protease activity (APIZYM test)

The APIZYM profiles of 8 *C. glabrata* and 2 *C. nivariensis* isolates were exhibited in Table 4.13. The common enzymes detected in *C. glabrata* isolates in this study were alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase and β-glucosidase. Almost similar types of enzymes were detected for *C. nivariensis* in this study (Table 4.13). Based on the amounts of APIZYM substrate metabolized by the isolates, three enzymes i.e., alkaline phosphatase, leucine arylamidase and acid phosphatase were considered the enzymes being produced abundantly in the *C. glabrata* isolates.

Table 4.13: Enzyme activity of *C. glabrata* and *C. nivariensis* isolates using APIZYM kit. The enzyme activity was graded from 0 (negative reaction) to 5 (maximum positive reaction), where: 1 corresponded to 5 nmoles, 2 to 10 nmoles, 3 to 20 nmoles, 4 to 30 nmoles and 5 to 40 nmoles of each APIZYM substrate metabolized by the isolates.

me name	Strain										*C.albicans ATCC90028*
	Cg 15	Cg 16	Cg 57	Cg 64	Cg 74	Cg 76	Cg 93	Cg 97	Cn 139	Cn 160	
Control	0	0	0	0	0	0	0	0	0	0	0
Alkaline phosphatase	5+	3+	5+	5+	5+	5+	5+	5+	3+	3+	5+
Esterase (C4)	4+	3+	4+	3+	3+	4+	1+	3+	2+	2+	4+
Esterase Lipase (C8)	3+	2+	4+	4+	3+	3+	3+	2+	2+	1+	3+
Lipase (C14)	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+
Leucine arylamidase	5+	3+	5+	5+	5+	5+	5+	5+	5+	5+	3+
Valine arylamidase	2+	1+	3+	4+	3+	3+	3+	3+	2+	1+	2+
Cystine arylamidase	2+	0	2+	3+	2+	3+	2+	4+	1+	2+	2+
Trypsin	0	0	0	0	0	0	0	0	0	0	2+
α-chymotrypsin	0	0	0	0	0	0	0	0	0	0	0
Acid phosphatase	4+	4+	5+	5+	5+	5+	5+	5+	4+	4+	4+
Naphthol-AS-BI-Phosphohydrolase	2+	1+	3+	3+	2+	3+	2+	4+	3+	3+	2+
α-galactosidase	0	0	0	0	0	0	0	0	0	0	0
β-galactosidase	0	0	0	0	0	0	0	0	0	0	0
β-glucuronidase	0	0	0	0	0	0	0	0	0	0	0
α-glucosidase	1+	0	0	2+	0	5+	4+	5+	2+	2+	2+
β-glucosidase	0	0	0	1+	0	4+	0	4+	1+	1+	1+
N-acetyl-β-glucosaminidase	3+	0	0	0	0	2+	0	0	0	0	4+
α-mannosidase	0	0	0	0	0	0	0	0	0	0	0
α-fucosidase	0	0	0	0	0	0	0	0	0	0	0

Cg= *Candida glabrata; Cn= C. nivariensis;* quality control strains: *C. albicans* ATCC90028

4.10.3 Biofilm formation

A total of 67 *C. glabrata* isolates were included in this test and the source of the isolates were derived from vagina (n =50), blood (n = 4), urine (n = 5), respiratory secretions (n = 1), and others (n = 7). The isolates were classified as weak, moderate and high biofilm producers based on the XTT $OD_{490\ nm}$ readings. A total of 16 isolates (24%) with $OD_{490\ nm}$ lower than the negative control (no microrooganism was inoculated) were considered negative in the biofilm formation. Isolates with $OD_{490\ nm}$ readings between 0.13-0.2 (n=20 isolates, 30 %) were considered weak biofilm producers, while isolates with readings between 0.2 - 0.3 (n=30 isolates, 44%) were considered as moderate biofilm producers. There was only one isolate (Cg 93 from a HVS sample, 2 %) with the OD readings of 0.3209, and thus it was considered as a high biofilm producer (Figure 4.16). The average XTT readings obtained for isolates investigated in this study (Table 4.14). All 4 *C. nivariensis* isolates formed less biofilm (OD readings 0.0823±0.0437) as compared to *C. albicans.*

The microscopic observations of the biofilm for a weak (Cg172, HVS (OD= 0.0071 ± 0.002), moderate (Cg 163, HVS OD=0.1487± 0.001) and high biofilm producer (Cg 93 OD= 0.3209 ± 0.001) after 48 hours of incubation are shown in Figure 4.17. Short filaments were observed for both Cg 163 and Cg 172. Pseudohyphae and hyphae were absent in majority of the isolates.

Table 4.15 shows the comparison of Pz* value for high and weak biofilm producers. The isolates with weak biofilm producers had significantly lower production of phospholipase (as indicated by the higher Pz* value) when compared to isolates with moderate biofilm producers ($P \leq 0.01$).

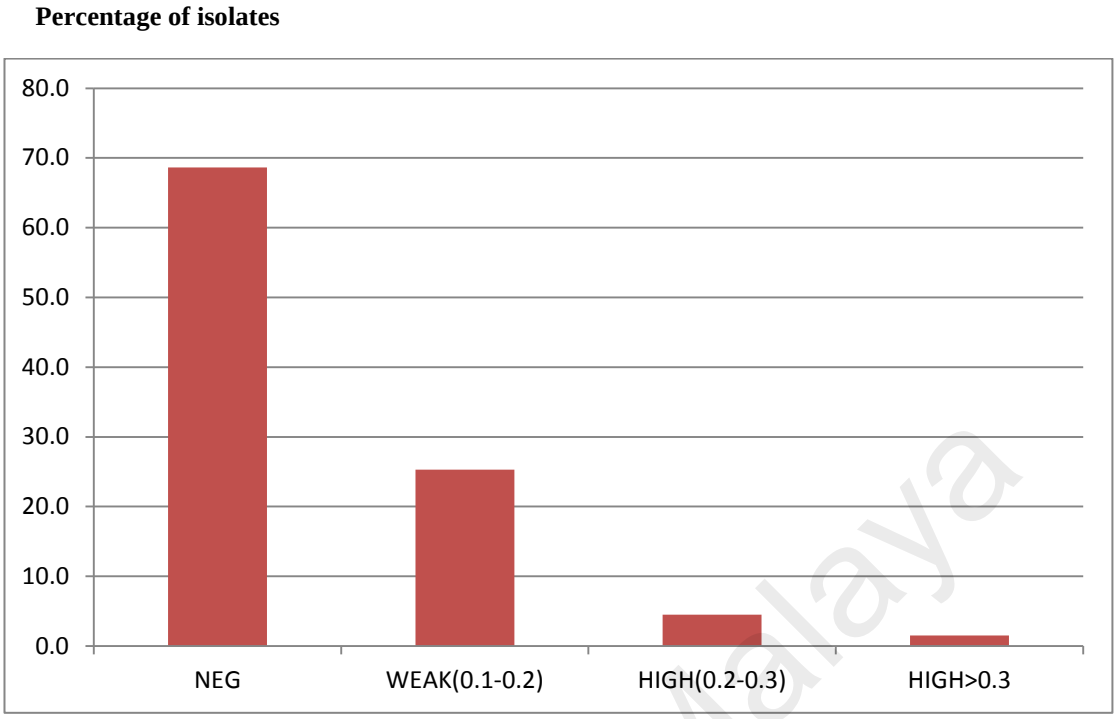
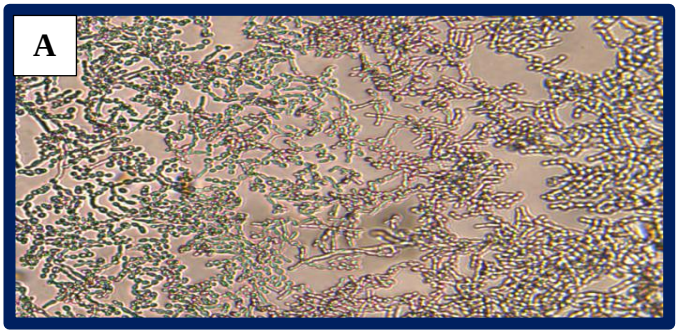


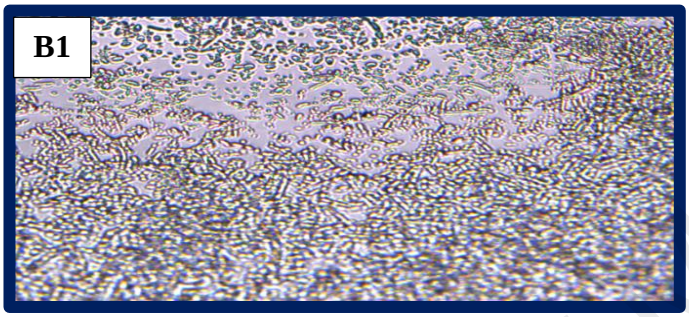
Figure 4.16: Comparison of the ability of *C. glabrata* in forming biofilms.

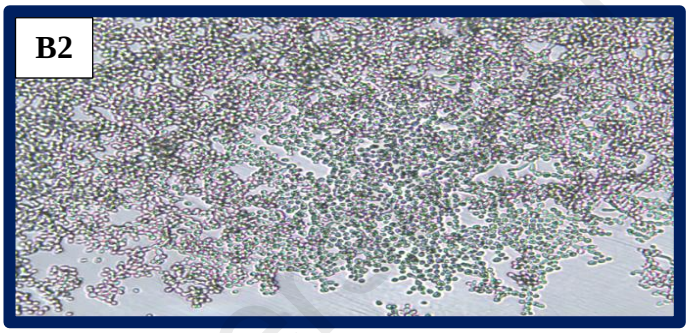
All isolates formed less biofilm as compared to *C. albicans.* The isolates were classified to weak, moderate and high biofilm producers based on XTT OD readings.(OD readings 0.2 were considered as weak biofilm producers, while isolates with readings of 0.2-0.3 were considered as moderate biofim producers).

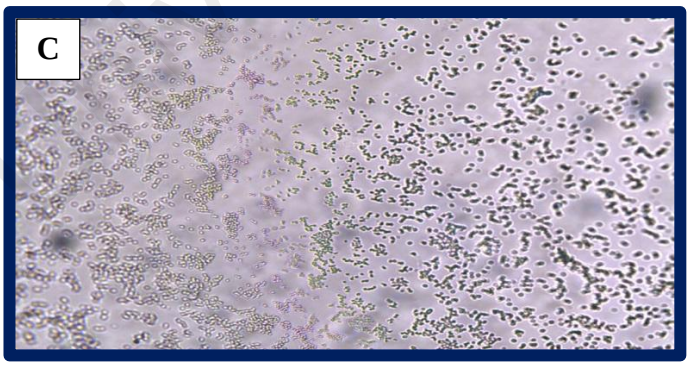
Table 4.14 Average XTT OD readings obtained from *C. glabrata* and *C. nivariensis* investigated in this study.

***C. glabrata* (n=67)**	**Range of OD readings**	**Average OD readings**
HVS (n=50)	0.0000 – 0.3209	0.0742 ± 0.0993
Urine (n=5)	0.0000 – 0.2507	0.0809 ± 0.1216
Blood (n=4)	0.0000 – 0.1966	0.0712 ± 0.1215
Sputum (n=1)	0.1931	_
Others (n=7)	0.0000 – 0.1996	0.0835 ± 0.1217
***C. nivariensis* (n=4)**	0.0000 – 0.1162	0.0437± 0.0617
***C. albicans* ATTC 90028**	0.3547	0.0050 ± 0.3596
Control negative Menadion + RPMI	0.1284	0.0001 ± 0.1290









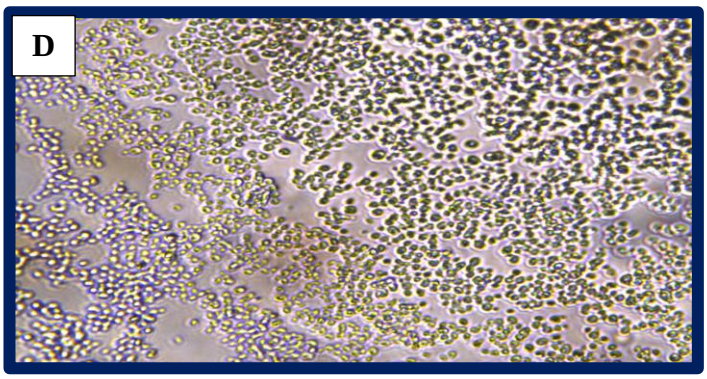


Figure 4.17: Images of biofilm produced by *C. glabrata* and *C. nivariensis* isolates after 24h of incubation (magnification X20).

A) Positive control, *C. albicans* ATCC 90028,

B1) *C. glabrata* moderate biofilm producer (Cg 163, HVS),

B2) *C. glabrata* weak biofilm producer (Cg 172, HVS),

C) *C. nivariensis* (Cn 139, blood culture),

D) *C. glabrata* negative biofilm producer (Cg76, B/C).

Table 4.15: Comparison of Pz* value for high and weak biofilm producers

Source of isolates	Average Pz* value for weak biofilm producer (n=number of isolates)	Average Pz* value for moderate biofilm producer (n=number of isolates)	P value
High vaginal swab	(n=21) 0.65 ± 0.56	(n=18) 0.50 ± 0.490	P ≤ 0.01
Urine	(n=2) 0.5± 0.51	(n=1) 0.42± 0.50	-
Blood	(n=1) 0.50 ± 0.55	(n=3) 0.41± 0.51	-
Others	(n=2) 0.51± 0.56	(n=3) 0.40± 0.44	-

Statistical analysis (Bivariate Correlation test, SPSS) was used to determine whether there was any significant difference in the phospholipase production of HVS *C. glabrata* isolates with weak and moderate biofilm producers.(If P≤0.01significantly related, If P>0.01not related). Note: SPSS analysis was only performed if there were more than five isolates.

CHAPTER 5

DISCUSSION

5.1 Identification of *Candida glabrata* using phenotypic and genotypic approaches

C. glabrata has been described as the second or third most common yeast species isolated from patients with vaginitis and invasive candidiasis. Accurate identification of *Candida* spp. from clinical specimens and antifungal susceptibility testing are important to facilitate optimal antifungal therapy and patient management. Two closely related species of *C. glabrata, i.e., C. nivariensis* and *C. bracarensis* have recently been identified as emerging pathogenic yeasts. (Alcoba *et al*., 2005; Correia *et al*., 2006).

A total of 185 *C. glabrata* isolates mostly from vaginal samples were collected in this study. Based on the proportion of the isolates collected in this study (Table 4.1), it appears that vaginal samples are the main source of the isolation for *C. glabrata*, as also reported by investigators in India (Deorukhkar *et al.,* 2014), Iran, Brazil and Yemen (Al-Mamari *et al*., 2014; Pádua *et al.,* 2003; Salehei *et al*., 2012). In these studies, the yeast collection concentrated only on vaginal candidiasis, and *C. glabrata* was the second predominant species after *C. albicans.*

5.2 Phenotypic identification of *C. glabrata*

Traditionally, the identification of *Candida* species from clinical specimens has been based on the morphological features and sugar fermentation patterns of the organisms. Conventional mycological methods that are used in the routine clinical microbiology laboratory rely upon a combination of morphological features coupled with the abilities of the organisms to ferment selected sugars or assimilate a variety of carbon and nitrogen sources (Borman *et al.,* 2008). In this study, sugar assimilation method used in the routine diagnostic laboratory was used to identify *C. glabrata* isolates (Ng *et al.,* 2001). Of seven sugars used in the panel (glucose, sucrose, trehalose,

maltose, galactose, cellobiose, arabinose), all *C. glabrata* isolates were found to assimilate glucose and trehalose sugars. However, this method alone is not sufficient for identifying C. *nivariensis* (Bishop *et al*., 2008).

Additionally, this study investigated the growth reaction of the *C. glabrata* isolates on CHROMagar. CHROMagar medium permits the detection of multiple yeast species from a single clinical specimen based on the colony color developed on the CHROMagar (Odds & Bernaerts, 1994; Pfaller, 1996). Despite the usefulness of Chromagar for differentiating *C. albicans*, *C. tropicalis, C. krusei* and *C. dubliniensis*, it is difficult to differentiate *C. guilliermondii*, *C. glabrata* and *C. humicolus* which produce purplish colony on CHROM agar (Peng *et al.*, 2007). In this study, a total of 129 (70 %) *C. glabrata* isolates produced purple colony, whereas 56 (30 %) of them produced white or creamy colonies (Table 4.1). Yeasts including *C. bracarensis, C. nivariensis, C. norvegensis* and *C. inconspicua* have been reported to produce white colonies on chromogenic media (Bishop *et al.*, 2008). It has been reported that white colony-producing yeasts are not frequently encountered in the clinical specimens (Murray *et al*., 2005; Odds & Bernaerts, 1994; Pfaller, 1996, Powell *et al.*, 1998). The higher percentage of *C. glabrata* producing purplish color than those producing white colors in this study, is in agreement with other studies (Adam *et al*., 2010; Bishop *et al*., 2008; Golia *et al*., 2013).

Different colony colours (white, pink or purple) produced by *C. glabrata* on chromogenic agar have also been reported (Ellepola & Morrison, 2005). The different color development of *C. glabrata* on CHROM agar may be caused by the production of different enzymes which react differently with chromogenic substrates in the CHROM agar medium, or spontaneous phenotypic switching which has been described in *C. glabrata*. *C. glabrata* has been reported to switch in a unique fashion between a number of phenotypes on agar containing cupric sulphate, a phenomenon referred to as "core

switching" (Lachke *et al*., 2000; 2002). Further study is required to explain the development of colony variants in C. *glabrata*, as this may help in the identification and understanding of the biology of this organism.

5.3 Amplification and sequence analysis of the internal transcribed spacer (ITS) region of *Candida glabrata*

Amplification of the internal transcribed spacer (ITS) region of *Candida glabrata* demonstrated that all *C. glabrata* isolates (regardless of their colony variants on Chromagar) produced amplified fragments of about 800 bp (Figure 4.6)**.** Mirhendi *et al.* (2011) reported that amplification of this region was accurate and reliable for separating *C. glabrata* from other yeast except for *C. nivariensis*, as both *C. nivariensis* and *C. glabrata* produced the same size of amplicon. The similar finding was also observed in this study (Figure 4.6).

The percentage sequence similarity of these isolates when compared to CBS 138 reference strain ranged from 98.7 to 100%. Hence, the intra species variation based on ITS sequences for *C. glabrata* is 1.3%. BLAST analyses show identical (100 % matching) sequences of 35 randomly selected isolates with at least one *C. glabrata* strain documented in the GenBank database (Table 4.6). Some of the ITS sequence types matched with those *C. glabrata* originating from different parts of the world, including Iran, China, Ireland, Germany, Denmark and Australia (Table 4.6). As greater than 1% substitution in the D1/D2 domain or ITS region usually represents separate yeast species **(**Fell *et al*. 2000; Kurtzman & Robnett, 2003; Scorzetti *et al*., 2002), further study is warranted to determine the species status of those isolates in G5, as the isolates demonstrated only 98.7% similarity with that of the reference strain, CBS 138 (Table 4.7). The differentiation of the isolates to nine ITS sequence types suggests the genetic heterogeneity of the isolates, of which majority consisting of vaginal isolates (Table 4.5).

5.4 Phenotypic and molecular identification of *C. nivariensis*

It has been reported that as *C. nivariensis* and *C. bracarensis* share many phenotypic characteristics with *C. glabrata,* they are likely to be misidentified as *C. glabrata* in the routine microbiology laboratory (Alcoba-Florez *et al.*, 2005; Borman *et al.*, 2008; Chowdhary *et al.*, 2010; Fujita *et al.*, 2007; Mirhendi *et al.*, 2011; Ruma-Haynes *et al* 2000; Wahyuningsih *et al*., 2008). Hence, molecular techniques including the singleplex PCR assay as described by Enache-Angoulvant *et al.* (2011) have been useful for definite identification of *C. glabrata*, *C. nivariensis* and *C. bracarensis*. In this study, the Singleplex PCR had successfully identified *C. nivariensis* from four isolates obtained from the blood specimen and vaginal swabs of two patients. No *C. bracarensis* was identified in this study.

The low prevalence of *C. nivariensis* in our clinical setting is in agreement with other published reports. *C. nivariensis* was found to be very rare (0.2% prevalence) in a global surveillance of *C. glabrata* clinical isolates from 28 countries in 2009 (Warren *et al.*, 2010). The yeast was not detected from two surveillance studies in Spain and Italy (Cuenca-Estrella *et al.*, 2011; Esposto *et al*., 2013). The incidence of *C. nivariensis* in the Asia region is considered rare too. A case of fungemia due to fluconazole-resistant *C. nivariensis* has been reported in Japan (Fujita *et al.*, 2007). In Indonesia, *C. nivariensis* has been isolated from a human immunodeficiency virus-infected patient who suffered from oropharyngeal candidiasis (Wahyuningsih *et al.*, 2008) whereas in India, two (0.5%) *C. nivariensis* isolates have been identified from the sputum and blood culture of patients (Chowdhary *et al*., 2010). In most studies, the clinical importance of *C. nivariensis* has been confirmed by the isolations of the yeasts from blood cultures and deep sterile body sites and the resistance to several antifungal drugs (Borman *et al*., 2008; Fujita *et al.*, 2007; Warren *et al*., 2010).

The characteristics of our *C. nivariensis* isolates matched the phenotypic features described by previous authors, i.e. growth of white colonies on CHROMagar and trehalose fermentation. The observation of only few positive reactions using commercial yeast identification kits such as ID 32 C and VITEK 2 yeast identification systems (Fraser *et al.,* 2012) as well as API20C AUX in this study, has made the identification of *C. nivariensis* challenging. In this study, API 20 C AUX test did not provide valid identification for the isolates. The API profiles (6000044) which were shared by the isolates demonstrated low probability for identification of *C. norvegensis*. Glucose was the only sugar assimilated by the isolates after 48h of incubation. Trehalose and glycerine assimilation were noted for the isolates only after 7 days of incubation. Strain Cn139 was the only isolate giving positive trehalose fermentation result by using GlabrataQuick™ Kit. However, further testing using a home-made trehalose test showed positive trehalose fermentation reactions for all isolates after three hours of incubation. Thus, *C. nivariensis* can be presumptively identified based on the production of white colonies on CHROMagar agar, microscopic features of small budding yeast cells, and a positive rapid trehalose test, as have been documented by other investigators (Alcoba-Florez *et al*., 2005; Bishop *et al.,* 2008; Correia *et al.,* 2006; Warren *et al*., 2010).

The ITS sequencing approach was able to discriminate *C. nivariensis* from *C. glabrata* and the finding was supported by the dendrogram as shown in Figure 4.9. Although sequence variation was detected amongst *C. nivariensis* isolates from different geographical regions (Table 4.8), the differentiation among these isolates is probably not conclusive, as reflected by the low bootstrap values on the dendrogram (Figure 4.9).

The increasing number of reports on *C. nivariensis* from various clinical sources suggests that the yeast may have a wider distribution globally than it is originally

thought. The low prevalence (1.1 %) of *C. nivariensis* in our clinical setting is in agreement with previous investigation (Chowdhary *et al*., 2010; Lockhart *et a*l., 2009).

5.5 Determination of genetic relatedness of *Candida glabrata* isolated from different anatomic sites

In spite of the increasing prevalence and importance of *C. glabrata,* little is known of the population structure and epidemiology of the yeast. Randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) allows a clear and rapid way to analysis yeast species (Andrighetto *et al*., 2000) and it has been frequently used for epidemiological investigations of *Candida* infections worldwide (Karaman *et al.* 2013; Lian *et al*. 2004; Marol & Yücesoy 2008; Muthig *et al.* 2010; refer to Paluchowska *et al*., 2014). In a Poland study (Paluchowska *et al*., 2014), RAPD typing of 17 *C. glabrata* isolates recovered from intensive care unit patients revealed 16 different genotypes. The Poland study was performed with different kinds of primers. High degree of genetic diversity was also demonstrated amongst *C. glabrata* strains originating from oral and vaginal specimens from Tunisia hospitals (Noumi *et al.* 2009). Although Ergon & Gülay (2005) observed a low degree of genetic diversity among their *C. glabrata* isolates; however, the authors suggested that primers used in their study were probably not appropriate for the typing of non-albicans species (Ergon & Gülay, 2005).

It has been reported that the strains classified as identical by one primer are not always classified as belonging to the same cluster when analysed by another primer (Bonfim-Mendonça *et al.,* 2013), hence, the data obtained with a number of oligonucleotides should be combined to generate higher discriminatory power (Soll, 2000). This is supported by Bonfim-Mendonça *et al.* (2013) who reported that the strains classified as identical by one primer are not always classified as belonging to the same cluster when analyzed by another primer. The results obtained in this study seems

to be in agreement with those reported previously, with the demonstration of 15 RAPD types for the *C. glabrata* isolates, when two primers [M13 and $(GTG)_5$] were used.

In this study, vaginal isolates were found to be genetically more diverse than those blood isolates, by looking at the number of fingerprinting patterns generated using both primers (Table 4.9). Only one RAPD type (I) was shared between the vaginal and blood isolates. There is evidence that *C. glabrata* from vaginal thrush could invade lavage fluid and blood in mouse models (Fidel *et al*., 1999). Also *C. glabrata* could penetrate deeper side of body easily if there is coinfection with *C. albicans* during vaginal infection (Alves *et al*., 2014). The isolation of *C. glabrata* from the placenta, serial blood and vaginal cultures of a patient with miscarriage history and recurrent vaginitis episodes has been reported (Posteraro *et al*., 2006).

As *C. nivariensis* is a newly identified organism, there is limited prevalence and genetic information of this yeast in most recent studies. The finding in this study demonstrates that differentiation of *C. nivariensis* from *C. glabrata* is feasible using RAPD analysis. Additionally, different RAPD pattern was generated for *C. nivariensis* in this study (Figure 4.11 and 4.12). In another study in India, the M13 PCR fingerprinting patterns for 4 *C. nivariensis* from vaginal and sputum isolates illustrated genotypic diversity in the *C. nivariensis* isolates (Sharma *et al*., 2013).

5.6 Determination of antifungal susceptibilities of *Candida glabrata*

None of the 80 *C. glabrata* and 4 *C. nivariensis* in this study was resistant against amphotericin B, fluconazole, voriconazole, caspofungin. However, 60% of the *C. glabrata* (n=80) were found to be clotrimazole-resistant, demonstrating MIC $\geq$ 0.5 µg/ml (MIC ranging from 0.03 - 8 µg/ml) (Table 4.11). In Malaysia, several studies have been conducted to determine antifungal susceptibilities of *Candida* species. Amran *et al.* (2011) reported that of 12 *C. glabrata* investigated for susceptibility

against amphotericin B, fluconazole, voriconazole, itraconazole and caspofungin using E test, only one was fluconazole-resistant. The study concluded that antifungal-resistance problem among clinically important *Candida* isolates in Kuala Lumpur Hospital was not worrying, however; continued antifungal-susceptibility surveillance are necessary to monitor the antifungal-susceptibility trends of *Candida* species. In another study using Sensititre Yeast One method, Santhanam *et al.* (2013) reported that resistance against posaconazole (47.1%), itraconazole (41.2%), and ketoconazole (58.8%) in *C. glabrata*. However, all *C. glabrata* isolates were found to be susceptible to caspofungin, amphotericin B, 5-flucytosine and voriconazole. Only one *C. glabrata* isolate was fluconazole-resistant while 9 (52.9%) were susceptible dose dependant. The authors stated that although the Sensititre yeast one method had been shown to have high agreement with CLSI method (Lombardi *et al*., 2004; Pfaller *et al*., 2006), a low agreement for susceptibility results with fluconazole for *C. glabrata* isolates MIC values (higher with the Sensititre Yeast One test compared to the CLSI reference method, was observed (Alexander *et al*., 2007), hence, it is importance to verify antifungal data by using different antifungal susceptibility methods.

Fluconazole-resistance in *C. glabrata* is not observed in this study. It has been reported that the prevalence of fluconazole resistance among *C. glabrata* isolates varies by country and region (Pfaller *et al.*, 2006). In China, approximately 14.3 % of *C. glabrata* isolates were resistant against fluconazole (Xiao *et al.*, 2015). In Iran, of 25 *C. glabrata* tested using disc diffusion method, low resistance rates against amphotericin B (1.1%), fluconazole (7.5%) and ketoconazole (4.3%) were reported (Mahmoudabadi *et al*., 2013). Clotrimazole (in the form of Canesten) is one of the most commonly prescribed drugs for vaginitis in the gynaecological clinics. In a previous study in Malaysia (Chong *et al.*, 2007), resistance to clotrimazole (MIC $\geq$ 0.5 μg/ml) has been reported in 6 of 7 isolates of *C. glabrata* from three patients with recurrent vaginitis.

Hence, the emergence of clotrimazole resistance in this hospital might have occurred as early as in 2003. The high occurrence of clotrimazole resistance may be responsible for clinical failure in recurrent vaginitis, as reported by some studies (Chong *et al.,* 2007; Sobel *et al.,* 2003). Clotrimazole-resistance was also noted in three *C. nivariensis* isolates (UMMC 145) obtained from one patient with recurrent vaginitis.

5.7 Determination of possible virulence factors of *Candida glabrata*

C. albicans and *C. glabrata* rank first and second in isolation frequency from cases of systemic candidiasis (Perlroth *et al*., 2007). However, little is known about the pathogenicity of *C. glabrata*. The ability of C. *glabrata* to cause invasive fungal infections suggests that the yeast may share some of the virulence properties of *C. albicans*. It has been reported that C. *albicans* follows an aggressive strategy to obtain nutrients for its survival; meanwhile *C. glabrata* seems to have evolved a unique strategy without causing severe damage to the host (Brunke & Hube, 2013). As both yeasts are known as successful commensals and pathogens of humans, comparison of the strategies undertaken by *C. glabrata* will provide better insights on the virulence properties of the yeasts and finding novel ways to fight candidiasis.

Hydrolytic enzymes such as secretory aspartyl proteinases, phospholipases and adherence of *Candida* species to host tissues have been regarded as the major determinants of the pathogenicity of *C. albicans* (Ghannoum, 2000; La Fleur *et al.,* 2006; Naglik *et al.,* 2004). The proteinase is generally known to facilitate microbial adhesion and tissue invasion and has been identified as one of the major pathogenic determinants in yeasts (Naglik *et al.,* 2004). Proteinase activity was undetectable in the *C. glabrata* isolates in this study, as shown by the lack of trypsin and α-chymotrypsin activity demonstrated in the APIZYM test. The absence of extracellular protease activity *in vitro* has also been reported by Kantarcioglu & Yucel (2002). However, a recent study from Malaysia (Tay *et al*., 2011) reported that 4 of 5 isolates

demonstrated low proteinase activity when a proteinase agar clearance assay was used. The use of different methods may have resulted with variable observation for proteinase production in *C. glabrata*. At the gene level, multiple orthologues of aspartyl proteases has been identified in the *C. glabrata* genome (http://cbi.labri.fr/Genolevures/elt/CAGL) (Kaur *et al*., 2005).

Invasion of the host cells by microbes entails penetration and damage of the outer cell envelope (Mohandas & Balla, 2011). Extracellular phospholipase has been recognized as a virulence factor for *C. albicans* and has been associated with membrane damage, adherence, and penetration of the host cells (Ghannoum, 2000). In a previous study in Malaysia (Tay *et al*., 2011), phospholipase activity was not detected in all five *C. glabrata* isolates. Similarly, none of the 4 *C. glabrata* isolates exhibited any phospholipase activity, as reported in a subsequent study in Malaysia (Chin *et al*., 2013). Phospholipase activity was detected from one of five *C. glabrata* isolates in Turkey (Kantarcioglu & Yucel, 2002). Four (28.6%) of 14 *C. glabrata* isolates from Turkey were reported to exhibit proteinase activity, while 5 (35.7%) had phospholipase activity (Kantarcioglu & Yucel, 2002). Although most studies reported the lack of phospholipase activity, weak production of phospholipase was observed from 88.7% isolates in this study (Table 4.12). This finding also confirms the genomic finding as three phospholipase B (PLB) genes have been reported in *C. glabrata* (Kaur *et al*., 2005). More sensitive assays are thus required for detection phospholipase activity of *C. glabrata*. Besides proteinase and phospholipase, several enzymes were identified from *C. glabrata* and *C. nivariensis* isolates in this study by using APIZYM kit (Table 4.13). The method of enzyme detection using in this approach is convenient and allows simultaneous detection of 19 enzymes from an isolate. The results obtained reveals strong production of alkaline phosphatase, leucine arylamidase and acid phosphatase by the *C. glabrata* and *C. nivariensis* isolates (Table 4.13).

Phosphatase is a type of enzyme which is used to free attached phosphoryl groups from other molecules during yeast digestion (Bull *et al*., 2002). Oshima (1997) reported that *Saccharomyces cerevisiae* has several phosphatases (including alkaline and acid phosphatase) with different specificities and cellular locations which are used in the uptake of inorganic phosphate (P_i) uptake. The acid phosphatase activity has been detected for each colony of yeasts, as the enzyme is located on the cell surface (Oshima, 1997). The same regulatory system also controls the synthesis of alkaline phosphatase, located in the cell envelope, whose activity is detected in yeast colonies (Oshima, 1997). As C. *glabrata* is genetically closely related to *S. cerevisiae* (Enache-Angoulvant *et al*., 2011), it is assumed that these enzyme activities are present in the yeast, and indeed, these enzyme activities were demonstrated using APIZYM tests in this study. Additionally, evidence is available that *C. glabrata* exhibits phosphatase activity during phosphate starvation conditions. The presence of a cryptic gene in *C. glabrata* which encodes a phosphate starvation-inducible acid phosphatase has been reported (Orkwis *et al*., 2010).

Acid phosphatase is known to enhance microbial survival by suppressing neutrophil respiratory burst and superoxide anion production in bacteria and parasites (Baca *et al*., 1993; Chen *et al.*, 1997; Reilly *et al*., 1996) The function of alkaline phosphatase is slightly different from acid phosphatase. Alkaline phosphatase is a hydrolase enzyme responsible for removing phosphate groups from many types of molecules, including nucleotides, proteins, and alkaloids and during the dephosphorylation period and is most effective in an alkaline environment (Tamás *et al*., 2002) However, their roles in the pathogenesis of *C. glabrata* are yet to be investigated.

Leucine arylamidase is believed to contribute to the invasion and play a role in *C. albicans* virulence by removing the N-terminal L-leucine from peptide substrates,

and as a result, facilitate the adhesion and penetration of *C. albicans* into the host tissues (Staniszewska *et al*., 2015).

The detection of lipase and esterase from *C. glabrata* in this study is also supported by other investigators: lipase has been detected from one of 14 isolates from Turkey (Atalay *et al*., 2015), while esterase has been detected from 33.3% of *C. glabrata* strains isolated from patients with diabetes in Iran (Fatahinia *et al*., 2015). This study provides basic information on the virulence traits of *C. glabrata*. Future investigation is required to determine the basic structures and functions of enzymes that play important roles in the initiation and progression of *C. glabrata* infections.

Biofilm formation is another virulence factor of *Candida* strains (Ramage *et al.*, 2009) which has gained the attention from the scientific community. Biofilms are described as surface-associated communities of microorganisms embedding within an extracellular matrix (Al-Fattani & Douglas, 2006; Silva *et al*., 2009; 2011). It has been reported that cells in the biofilm are more resistant to antifungal treatment. The resistance to antimicrobial agents can be achieved by delaying the penetration of the antimicrobial agent (Donlan & Costerton, 2002).

Amongst non-albicans *Candida* pathogenic yeasts, *C. glabrata* has been reported to produce multilayered biofilm structures in host tissues and abiotic surfaces (Iraqui *et al*., 2005). EPA6 (Epithelial Adhesin gene6) gene has been known to encode the main adhesin involved in biofilm formation in this opportunistic yeast (Iraqui *et al*., 2005; Riera *et al.*, 2012). Of 67 *C. glabrata* isolates included in this study for assessment of biofilm formation, 16 isolates (24%) were negative, 20 (30%) isolates were considered as weak and 30 isolates (44.5%) were moderate biofilm producers.

Only one (1.5 %) HVS isolate was considered as high biofilm producer. All 4 *C. nivariensis* isolates formed less biofilm as compared to *C. albican*s, of which two

demonstrated weak biofilm production. In a previous study (Silva *et al.,* 2009), it has been reported that *C. glabrata* biofilms produced less total biomass and was less able to form biofilms than *C. parapsilosis* and *C. tropicalis* strains. However, the matrices recovered from *C. glabrata* strains had higher amounts of both proteins and carbohydrates which could be related to potential virulence of this species (Silva *et al.,* 2009). It has been described that the biofilms of *C. glabrata* presented as a multilayered structure with blastoconidia intimately packed, for some strains, and for others as a biofilm composed of cell clusters, which is also observed in this study (Figure 4.17). The study also confirms previous findings in Malaysia (Tay *et al.,* 2011) whereby no obvious filamentation was observed for C. *glabrata*. The finding that the weak biofilm producers demonstrated significantly less phospholipase activity when compared to those produced by the high biofilm producer (Table 4.15) is yet to be confirmed with testing of a large number of isolates.

5.8 Study limitation

Information about the isolates (source of isolates, reason for hospital admission and specimen type) were obtained based on laboratory records at UMMC. The full clinical records of the patients should be reviewed in order to provide information on the medical history, duration of infection, effect of antifungal agent used for the yeast infection. Additionally, *C. glabrata* and *C. nivariensis* reference strains could be included in this study for comparison with the local strains. However, these strains were not available at the time of study.

Although RAPD analysis has been recommended for the investigation of *Candida* epidemiology at a local level (Saghrouni *et al*. 2013), it may result in low interlaboratory reproducibility. The data should be interpreted with caution, and be supplemented with other genetic techniques such as multilocus sequence typing.

CHAPTER 6

CONCLUSION

Molecular techniques are excellent tools for identification of new species and strain typing of yeasts. In this study, *C. glabrata* DNA was extracted from each isolate and subjected to testing using a singleplex PCR assay for discrimination between *C. glabrata, C. bracarensis* and *C. nivariensis*, as described by Enache-Angoulvant *et al.* (2011). This study reported for the first time in Malaysia the identification of four isolates of *C. nivariensis*. The identity of the isolates was confirmed based on sequence analysis of the yeast internal transcribed spacer (ITS) region and D1D2 domain. The low prevalence (1.1 %) of *C. nivariensis* in the *C. glabrata* isolates is in agreement with several studies reported in other geographical regions.

Based on the ITS sequence data obtained from 35 randomly selected *C. glabrata* isolates, a total of 9 sequence types were identified. All *C. glabrata* and *C. nivariensis* isolates were susceptible to amphotericin, caspofungin, fluconazole, and voriconazole. Randomly amplified polymorphic DNA (RAPD) analysis of 58 *C. glabrata* isolates using two single primers [M13 and $(GTG)_5$] identified five and six genetic clusters, respectively. The further differentiation of the isolates to 15 RAPD types exhibits the genetic heterogeneity of *C. glabrata* isolates. *C. nivariensis* isolates formed a unique genetic cluster as compared to *C. glabrata* isolates. *C. glabrata* was regarded less virulent compared to *C. albicans* probably due to the very weak phospholipase activity and biofilm production, as demonstrated by the isolates investigated in this study.

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List of publication and poster presented

PUBLICATIONS

1. Sun Tee Tay, **Azadeh Lotfalikhani**, Negar Shafiei Sabet, Sasheela Ponnampalavanar, Sofiah Sulaiman, Shiang Ling Na, Kee Peng Ng.

Occurrence and Characterization of *Candida nivariensis* from a Culture Collection of *Candida glabrata* Clinical Isolates in Malaysia. Mycopathologia. 8(5):DC01-4. doi: 10.7860/JCDR/2014/7434.4311, May, 2014.

2. **Lotfalikhani** et al. Genetic heterogeneity, antifungal susceptibility and enzymic profiles of Malaysian clinical isolates of *Candida glabrata* (submitted for review).

POSTER PRESENTATIONS

1. Molecular identification and characterization of Malaysian isolates of *Candida glabrata* and *Candida nivariensis*. National postgraduate Seminar, University of Malaya, Kuala Lumpur, Malaysia, 11 July, 2012.

2. Genetic characterization and antifungal susceptibilities of *Candida glabrata* and *C. nivariensis* isolated from clinical specimens. 9th International Symposium on Antimicrobial Agents and Resistance (ISSAR 2013), Kuala Lumpor, Malaysia, 13-15 March, 2013.