Invasive Yeast Infections –

Understanding the Current Scene

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Statement of Originality and Responsibility

The work contained in this thesis is completely original and has not been submitted to any university for another degree. The series of studies described were carried out from 1999 – 2009 under the auspices of the Department of Microbiology, the Chinese University of Hong Kong, and research grants awarded from the Chinese University of Hong Kong. I was responsible for the study design, application for funding support, monitoring of experimental work, analysis of data generated, finalizing the results and preparation of manuscripts for publications and presentations.

Précis

A. Thesis Outline

This thesis describes a series of studies conducted from 1999 to 2009 that aimed at understanding the clinical impact of invasive yeast infections in Hong Kong. It focuses on the epidemiology, pathogenesis, diagnosis and antifungal resistance of these important but often neglected infections. With such understanding, it is aspired to help reduce the morbidity and mortality of these often fatal diseases.

Part I. The thesis starts with the background development of clinical mycological research. Biology of the pathogenic yeast species is discussed. This is followed by the descriptions of the clinical infectious diseases and a review of currently available diagnostic methods and treatment modalities.

Part II. This part describes the studies in detail. The first three studies aimed at reviewing the invasive yeast infections in Hong Kong, including *Candida* species, *Pneumocystis jirovecii* (formerly *Pneumocystis carinii*) and *Cryptococcus neoformans*. As *Candida* is the commonest invasive yeast encountered, this led to the subsequent studies focusing on various aspects of *Candida* infections. The next 2 studies aimed at identifying highly polymorphic genetic regions and exploring novel molecular methods to assess the genetic relatedness of pathogenic *C. albicans* isolated from nosocomial infections. It is followed by another two studies which aimed to develop and to apply non-culture-based diagnostic methods for the rapid diagnosis of invasive *Candida* infections. Susceptibilities of orally available antifungal agents and over-the-counter preparation against *Candida* species were then assessed. The last study explored the pathogenesis of vascular catheter associated candidaemia and the contribution by agglutinin-like-sequence (*ALS*) genes of *C. albicans* on catheter adherence.

Part III. This part summarizes the major findings of the previous local studies. Further discussions and conclusions is made with emphasis on how these results advance our understanding of these invasive fungal diseases, and its

implication on earlier diagnosis, treatment, prevention of these fungal infections and future research directions.

B. Background

Yeasts are unicellular fungi. Among the approximately 1500 recognized species, a few of them can cause human infections. Traditionally, yeast infections were given little attention, as they were likened to mild superficial infections, such as mucocutaneous candidiasis, and thus deserved little attention. With the advent of immunosuppressive therapies and AIDS, the unwelcome scene of invasive fungal infections has emerged. Pathogenic yeasts that are capable of causing invasive diseases in immunocompromised patients include the *Candida* species, *Pneumocystis jirovecii* (formerly *Pneumocystis carini*), and *Cryptococcus neoformans*. These yeasts are opportunists, and their infection syndromes often share the predicaments of difficulty with culture-based diagnosis, delay in initiation of appropriate antifungal treatment, intolerance of toxic antifungal agents, lack of preventive strategies, and controversies with secondary prophylaxis in patients other than AIDS. All these result in the notoriously high morbidity and mortality rates of invasive yeast infections. On the other hand, the research field of medical mycology has evolved rapidly lately. The antifungal arsenal has strengthened with the newly available echinocandins and second-generation triazoles. The expanded armamentarium not only improves patient care, but also stimulates renewed interest in antifungal susceptibility testing and research into resistance mechanisms. In addition, difficulty with culture-based diagnostic had prompted research into non-culture based techniques with molecular diagnostics and metabolite detections. Studies into the genomic relatedness had also shown possible nosocomial acquisition of the yeasts in the healthcare settings, and potential impact on infection control measures. The availability of the *Saccharomyces* and *C. albicans* genome database has allowed exploration into the molecular pathogenesis of fungal infection. In particular, the molecular mechanism of adhesion of *C.* organisms onto intravascular catheters, which is the first step in the sequence of events leading to catheter colonization and subsequent disseminated infections. Research into the local and current scene of

invasive yeast infections, will not only deepen our understanding of the diseases, but also guide us onto further clinical and basic mycological research areas that are of relevance in providing quality patient-care.

C. Studies on the Disease of Invasive Infections by Yeasts.

Study One: Cryptococcus neoformans infections in a setting with low HIV prevalence.

Background. *Cryptococcus neoformans* is an encapsulated yeast capable of producing life-threatening meningitis and disseminated infections, especially in the immunocompromised patients such as those with HIV infections. In a locality where the HIV prevalence is low, the disease is often not suspected, leading to a delay in diagnosis and institution of appropriate antifungal treatment. Thus, we aimed to analyse the disease in our locality.

Methods. A retrospective review was conducted on all patients' records with microbiological confirmation of *Cryptococcus neoformans* infection at the Prince of Wales Hospital during the period 1993 to 2008.

Results summary and discussion. A total of 37 non-duplicate patients were identified during the period. Twenty-seven of them had meningitis, 6 with blood stream infection, 2 with pneumonia, 1 with retinitis and 1 with CAPD associated perotinitis. In 5 patients, the diagnosis was not diagnosed ante-mortem. Overall mortality rate was 43%. As the disease appears to be uncommon in our locality, a high index of suspicious is required.

Study Two: Pneumocystis jirovecii (carinii) pneumonia in a tertiary referral hospital.

Background. *Pneumocystis jirovecii* is an opportunistic fungus capable of causing life-threatening pneumonia in patients with impaired cellular immunity and HIV infection. However,

much less is known about *Pneumocystis jirovecii* pneumonia (PCP) in non-HIV-infected patients. Since Hong Kong has a very low prevalence of HIV infection, the spectrum of PCP infection may be different. The aim of this study was to analyse the characteristics of patients suffering from PCP.

Methods. A retrospective case review was conducted on patients suffering from PCP at the Prince of Wales Hospital in Hong Kong from 1993 to 2008. Cases of PCP were identified from the laboratory log from the Microbiology Department. The laboratory utilized a toluidine blue O staining method to identify the fungus.

Results summary and discussion. Thirty patients were identified during this period. Sixteen (53.3%) were co-infected with human immunodeficiency virus (HIV). The remaining non-HIV-infected patients had underlying diseases including post-renal transplant recipients, haematological malignancies, auto-immune diseases, renal diseases, hepatocellular carcinoma and congenital cytomegalovirus disease. Overall crude mortality was 26.7%. Chemoprophylaxis should be considered in populations at risk.

Study Three: Invasive Candida blood stream infections from 1993 to 2008 – a retrospective review.

Background. *Candida albicans* and *Candida* non-albicans are commonly found as commensals on the human body. Hence, its significance as opportunistic pathogens is often neglected. Thus, a retrospective review was undertaken to analyse the clinical characteristics of invasive blood stream infections caused by *Candida* species.

Methods. A retrospective case review was conducted on patients suffering from invasive *Candida* blood stream infections at the Prince of Wales Hospital in Hong Kong from 1993 to 2008. Cases were identified from the laboratory log from the Microbiology Department. During this period, the laboratory utilized automated blood culture system for blood culturing.

Results summary and discussion. A total of 480 candidaemic episodes were identified, involving 478 patients. Among them, 259 (52.2%) were *C. albicans*. For the remaining *Candida*

non-albicans species, 60 (12.1%) were *C. parapsilosis*, 105 (21.2%) were *C. tropicalis*, 50 (10.1%) were *C. glabrata* This is consistent with the global trend that *C. albicans* is no longer the predominant organisms found in invasive diseases. Other *Candida* species with variable amphotericin and azoles susceptibilities had emerged. This has impact on the most appropriate choice of antifungal used.

D. Studies on Genetic Relatedness and Epidemiological Typing

Study four: Identification of polymorphic regions in the 18S and 25S rRNA genes for the epidemiological typing of Candida albicans isolates.

Background. *C. albicans* often presented with life-threatening invasive infections in immunocompromised patients. As these patients were often given broad spectrum antibiotics beforehand, it was also observed that they often became extensively colonized with *C. albicans*, such as from tracheal aspirates, catheterized urines or wound swabs. Thus, the question was often raised on whether such *C. albicans* colonization, could predate the advent of invasive diseases in these highly susceptible hosts.

Methods. The 18S and 25S rRNA genes of *C. albicans* were screened for genetic variations among a collection of 47 clinical isolates in order to identify highly polymorphic regions for epidemiological typing of *C. albicans*. The two genes were each divided into five regions for polymerase chain reaction amplification. Genetic variations were detected by the single stranded DNA conformation polymorphism techniques.

Results summary and discussion. Genetic variations were observed in three out of the ten regions studied. A combined total of twenty patterns were observed among the 47 clinical isolates. Such technique shall facilitate the investigation of genetic relatedness of the organisms. The patterns observed could provide a basis of understanding for the acquisition and development of *C. albicans* colonization and invasion.

Study five: Highly discriminating and reproducible typing of Candida albicans by a novel low-stringency random amplification of polymorphic DNA (RAPD) approach.

Background. A novel molecular typing technique is explored and assessed on its usefulness in discerning the genetic relatedness of clinically isolated *C.albicans* strains.

Methods. A specially designed PCR protocol was devised in which a range of annealing temperatures (40°C – 50°C) in each amplification cycle greatly enhanced the efficiency of producing a RAPD profile through reduction of primer-target annealing stringency during PCR. This amplification strategy provided optimal annealing temperatures for all potential target sites so as to maximize the number of PCR products to be generated in a RAPD reaction, the patterns of which are subsequently depicted by polyacrylamide gel electrophoresis and silver staining.

Results summary and discussion. This typing method was found to yield a highly unique and reproducible RAPD banding pattern for each isolate, thus providing extremely good resolution for delineating genetic relatedness and possible routes of transmission of clinical *C. albicans* isolates.

E. Studies on Non-culture-based Diagnostic Methods

Study six: Rapid Identification of medically important Candida at species level using polymerase chain reaction and single-strand conformational polymorphism.

Background. With the emergence of fluconazole and amphotericin B resistance, the rapid identification of fungi to species level is of clinical relevance in guiding appropriate antifungal therapy. A molecular technique is devised to identify *Candida* organisms to species level rapidly.

Methods. A molecular method utilizing single-strand conformational polymorphism (SSCP) to delineate different patterns on a 260-bp amplicon from the 28S rRNA gene from six

medically important *Candida* species was developed.

Results summary and discussion. The SSCP banding patterns obtained from a total of 52 isolates were sufficiently unique to allow distinction between the species, thus indicate a high level of specificity. This method of PCR-SSCP can provide a simple and specific method for the rapid identification of medically important *Candida* to species level.

Study seven: Development and application of a rapid diagnostic method for invasive candidiasis by the detection of D-/L-arabinitol using gas chromatography/mass spectrometry.

Background. D-arabinitol is a five-carbon sugar alcohol (pentitol) produced by many pathogenic yeast species, whereas humans are capable of producing both D- and L-arabinitol. A rapid non-culture-based diagnostic method utilizing D-/L-arabinitol (DA/LA) ratios as a chemical marker of invasive candidiasis was developed and explored.

Methods. The enantiomers-ratios measurement was made possible by the use of gas chromatography coupled with mass spectrometry (GC/MS). Possible results interference with healthy subjects with or without *Candida* colonization was explored.

Results summary and discussion. The difference in DA/LA ratios between the candidaemic patients and the non-candidaemic patients were statistically significant ($P < 0.01$) in both serum and urine samples. The DA/LA ratios were not significantly affected in patients with oral or vaginal candidiasis and candiduria. Therefore, the measurement of DA/LA ratios presents a possible addition to the armamentarium of diagnostic tools in invasive *Candida* infections.

F. Studies on Susceptibilities Testing

Study eight: In vitro activity of fluconazole and voriconazole against invasive Candida isolates in patients with candidaemia from 2001 – 2008.

Background. Antifungal treatment ranges from conventional amphotericin B to the new echinocandins. Azole agents provide an alternative choice because oral formulation is available. Fluconazole and voriconazole has been used in the treatment of candidaemia for several years, thus, it is important to monitor for the trend of antifungal resistance.

Methods. Non-duplicate *Candida* isolates collected from candidaemic patients from 2001 to 2008 were tested against fluconazole and voriconazole in accordance to the NCCLS(CLSI) standard M44-A by disk diffusion method.

Results summary and discussion. A total of 143 *C. albicans*, 37 *C. parasilopsis*, 38 *C. glabrata*, and 57 *C. tropicalis* isolates were available for testing. Among which, only 6 *C. albicans* (4.2%) revealed non-susceptibility to fluconazole.and/or voriconazole. *C. parapsilosis*, *C. tropicalis* and *C. glabrata* showed non-susceptibility to fluconazole at 0%, 14% and 39.5% respectively. That of voriconazole was 0%, 7% and 2.6 % respectively. Thus, even though fluconazole has been under extensive use for many years, its resistance rate against *C. albicans* and *C. parapsilosis* remains very low. For other species, susceptibility results can be variable, suggesting susceptibility test may be needed in case of suboptimal clinical response. Voriconazole has slightly better effect than fluconazole.

Study nine: In vitro susceptibility testing of vaginal C. albicans isolates against boric acid and its possible antifungal mechanisms.

Background. Boric acid therapy for the treatment of vulvovaginal candidiasis was described more than 30 years ago. It is prescribed as a vaginal capsule. It is envisaged that this agent may be useful in treating patient heavily colonized with *Candida*, and thus act as a preventive management modality. In this study, we aimed at evaluating the susceptibility profile of vaginal *C. albicans* isolates to boric acid by the use of agar dilution method.

Methods. A total of 12 vaginal yeast isolates of *C. albicans* were tested together with type strains of *C. albicans*. Boric acid in different concentrations was incorporated into Petri plates

with RPMI 1640 medium with final concentrations ranging from 0.015-32 mg/L. The minimum inhibitory concentration (MIC) value was recorded as the lowest concentration that inhibited visible growth after 24h of incubation; and also again after 48h of incubation. Biofilm and hyphal formation in boric acid treated yeasts were assessed *in vitro*.

Results summary and discussion. At 24 hour, all vaginal isolates and types strains of *Candida* demonstrated an MIC of >32mg/L against boric acid, with the exception of *C. krusei* ATCC 6258 which showed an MIC of 32mg/L. By 48 hours, the *C. krusei* ATCC 6258 also demonstrated an MIC of >32mg/L. Inhibitions of biofilm and hypphal formation were observed *in vitro*. Despite the high MIC values recorded, boric acid is administered topically at a concentration much higher than was used in our test system. It is thus plausible that the fungus be inhibited by the highly concentrated drug *in vivo*, resulting in apparent clinical cure.

G. Studies on ALS gene family in invasive Candida isolates

Study ten: Detection of ALS1, ALS5 and ALS6 genes in invasive Candida albicans isolates.

Background. Adhesion onto catheters is the first step leading to subsequent infections. The *ALS* genes family encodes cell wall proteins which had been demonstrated to possess adhesion properties. The aim of this experiment was to investigate the presence of *ALS1, ALS5* and *ALS6* genes in invasive clinical *Candida* isolates. .

Methods. Candidaemic isolates from patients with or without positive growth from vascular catheter were chosen. Eleven catheter and 8 non-catheter-related *C. albicans* isolates were selected.

Results summary and discussion. Five isolates were positive for all three *ALS* genes. *ALS1* was detected in 9 out of 11 *C. albicans isolates*. The remaining isolates showed various combination of *ALS1*, *ALS5* and *ALS6*. None of the non-catheter related isolates had *ALS1* or *ALS6*.

Although *ALS1* and *ALS6* genes were plausible explanation for catheter associated candidaemia, they were not invariably present. Other virulence factors should be considered also.

H. Conclusions

The studies described in this thesis have generated a spectrum of information on invasive yeast diseases. *Candida* species constitute the majority of the infections. Molecular techniques have enabled us to unravel the pathogenic mechanism involved, to understand and to detect possible nosocomial acquisition of the infections. Coupled with metabolites detection, rapid diagnosis and species identification are possible. Antifungal susceptibility surveillance would allow rational choice of the agents, and to monitor the emergency of resistance.

Publications and Presentations

PUBLICATIONS

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2. M Hui, SW Cheung, ML Chin, KC Chu, RCY Chan, AF. Cheng. Development and Application of a Rapid Diagnostic Method for Invasive Candidiasis by the Detection of D-/L-Arabinitol Using Gas Chromatography/Mass Spectrometry. *(Diagnostic Microbiology and Infectious Disease 2004,49 117-123)*

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15. M Hui, M Eastel, C Fu, WH Lam, CY Chan. In vitro activity of fluconazole and voriconazole against invasive *Candida* isolates in patients with candidaemia from 2006 – 2007 in Hong Kong. *(11th Western Pacific Congress of Chemotherapy and Infectious Diseases, Taiwan, 2008)*

16. M Hui, M Eastel, CLH Fu, WH Lam, CY Chan. *Cryptococcus neoformans* infections in non-HIV-infected patients: predictors of mortality. *(26th International Congress of Chemotherapy and Infection, Toronto, Canada, 2009.)*

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List of Abbreviations

5-FC	5-fluorocytosine
5-FU	5-fluorouracil
ABCD	amphotericin B colloidal dispersion
ABLC	amphotericin B lipid complex
AIDS	acquired immunodeficiency syndrome
ALS	agglutinin like sequence
AML	acute myeloid leukaemia
API	analytical profile index
Ara-C	cytarabine
ATCC	American type culture collection
AUC	area under curve
bp	base pairs
BDG	β-D-glucan
°C	degree Celsius
CAPD	continuous ambulatory peritoneal dialysis
CD4	cluster of differentiation 4
CFU	colony forming unit
CGB	canavanine glycine bromothymol blue
CHOP	cyclophosphamide, doxorubicin, vincristine, prednisolone
CLSI	Clinical and Laboratory Standards Institute
C_{max}	peak concentration
CMV	cytomegalovirus
CNS	central nervous system
CSF	cerebrospinal fluid
CT	computed tomography
CYP	cytochrome enzymes
DA	D-arabinitol
dL	deciliter
DM	diabetes mellitus
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
EDTA	ethylenediaminetetraacetic acid
EIA	enzyme immunoassay
ELISA	enzyme linked immunosorbent assay
ESRF	end stage renal failure

FEP	tetrafluoroethylene and hexafluoropropylene polymer
FNAC	fine needle aspiration cytology
g	gram
GC	gas chromatography
GM	galactomannan
G6PD	glucose-6-phosphate dehydrogenase
HAART	highly active antiretroviral therapy
HCL	hydrochloric acid
HCW	healthcare worker
HIV	human immunodeficiency virus
hr	hour
ICU	intensive care unit
iv	intravenous
KCl	potassium chloride
kg	kilogram
LA	L-arabinitol
LAMB	liposomal amphotericin B
mg	milligram
$MgCl_2$	magnesium chloride
MIC	minimum inhibitory concentration
min	minute
mL	milliliter
mm	millimeter
MRSA	methicillin resistant *Staphylococcus aureus*
MS	mass spectrometry
NCCLS	National Committee for Clinical Laboratory Standards
NHL	Non-Hodgkin's lymphoma
NNIS	National nosocomial infections surveillance
NPC	nasopharyngeal carcinoma
ng	nanogram
p	probability
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered solution
PCP	*Pneumocystis jiroveci* pneumonia
PCR	polymerase chain reaction
pg	picogram
RAPD	random amplification of polymorphic DNA
RFLP	restriction fragment length polymorphism

RIA	radioimmunoassay
RNA	ribonucleic acid
SD	standard deviation
SLE	systemic lupus erythematosus
sec	second
SSCP	single strand conformation polymorphism
TPN	total parenteral nutrition
TURP	transurethral resection of prostate
µg	microgram
µL	microlitre
µm	micrometre
µM	micromolar
WBC	white blood cell
YNB	Yeast nitrogen broth
yr	year

PART I: BACKGROUND

Chapter 1: Introduction

Clinical mycology is an important, but neglected specialty. The fungal organisms involved are often considered as contaminants and receive little attention. The problem is compounded further by the confusion in terminology and taxonomy. Researchers in basic sciences, clinical sciences, botany and industry gave different names to the same organism. Take *Candida albicans* as an example; it was called *Oidium albicans*, *Syringospora robinii*, *Mycoderma vini*, *Saccharomyces albicans*, *Monilia albicans* and *Candida albicans* by different researchers (Barnett 2000). This resulted in poorly defined clinical descriptions of moniliasis, candidosis and candidiasis (Hurley 1964; Odds 1987, 1992). Early scientific literature was also difficult to follow due to the plethora of languages used for publication. For example, Otta Jirovec, the Czech parasitologist who described human cases of pneumocystis infections, had published on it in Czech, Dutch, French, German, Polish, Russian but not English during the 1950s and 60s. This probably contributed to the lack of recognition of his work until recently (Frenkel 1999).

In the 1960s, when antibiotics and vaccines were then seen as great powers that will put an end to pestilence, resources were shifted to other chronic illnesses. Progress and incentive in microbiological and infectious diseases researches wizened (Sassetti 2007). Clinical mycology was no exception (Homei 2008).

On the other hand, biologists and chemists supported by the brewery and winery industries, had made important advancement in the understanding of fermentation and sugar utilization in fungi such as *Aspergillus oryzae* (the making of Japanese saké), *Saccharomyces carlsbergensis* (the making of lager), and *Saccharomyces cerevisiae* (the making of ale) (Barnett 2001). The characterization of these yeasts, had made it possible for *Saccharomyces cerevisiae* to become an important model organism in biological research such as molecular biology, genomic and proteome research. It is also the first eukaryotic organism to have its genome completely sequenced (Goffeau 1996).

In the wake of the HIV/AIDS epidemic in the 1980s, together with the advancement in cancer chemotherapy, intensive immunosuppressive therapies and transplant technologies, the associated opportunistic infections had revived renewed interests. Thanks to the pioneering work of early researchers, the scientific community had started to agree on the taxonomy of some of the pathogenic fungi. The genus *Candida* was adopted in lieu of *Monilia*. Standardized identification of *Candida* species with the use of absorbed antisera was also possible (Barnett 2008). Progress in clinical mycology research had slowly, but steadily picking pace. In

terms of diagnostics, improved antigens, metabolites and nucleic acid detection methods had begun to emerge. In treatment options, on top of liposomal amphotericin B, new azole agents such as voriconazole and posaconazole; and the novel class of echinocandin targeting fungal cell wall synthesis, had successfully made it through clinical development. With the completion of the genome sequence of *C. albicans* (Jones 2004) and *Aspergillus fumigatus* (Nierman 2005), it is now possible to explore into the molecular mechanism of fungal resistance, to identify possible virulence or pathogenic gene families, and to access their functional roles in human diseases (Costanzo 2006; Nguyen 2004; Odds 2004).

In the midst of the Fungal Kingdom, several yeast organisms are of particular concern. The *Cryptococcus neoformans* and *Pneumocystis jirovecii* (formerly *Pneumocystis carinii*) infections had earned their fame as AIDS defining diagnosis (CDC 1992). This not only highlights their important roles in AIDS patients, but also as important opportunistic pathogens in other immunocompromised patients as well (Ewig 1995; Kiertiburanakul 2006; Pappas 2001). The *Candida* species are also recognized as one of the commonest pathogens in blood stream infections, notably in patients requiring intensive care and central vascular catheterisation (Horn 2007; Tortorano 2004). These have far reaching implications. In localities

where HIV/AIDS are not considered or conceived to be prevalent, the disease presentation could well be different when compared with other countries. Many a times, the lack of awareness could lead to delay in diagnosis and institution of appropriate therapy. In invasive *Candida* infections, the lack of attention to infection control in handling vascular catheters and the reluctance to remove the central venous catheter often lead to failure of treatment (Kibbler 2003). The therapy also needs to be tailored according to the pharmacodynamics and pharmacokinetics of the population concerned. Ethnic differences such as cytochrome P450 activities and prevalence of glucose-6-phosphate dehydrogenase deficiency will have an impact on the choice and monitoring of treatment (Chan 1997; Fung 1969; Wang 2009).

With all these in mind, this thesis attempted to explore and to understand the invasive infections caused by these fungal pathogens.

Chapter 2:
Biology

2.1 *C. albicans* and related species.

The genus *Candida* comprises of more than 100 species. Among them, common invasive human pathogens are *C. albicans*, *C. glabrata*, *C. tropicalis* and, *C. parapsilosis.* Occasionally *C. krusei*, *C. guilliermondii, C. dubliniensis, C. lusitaniae* and rarely other species are implicated in human diseases. Several international surveillance studies on *Candida* blood stream infections had shown that *C. albicans* was responsible for roughly half of the candidaemic cases (Pfaller 1998, 1999, 2000, 2001; Messer 2006). A shift in species distribution, mainly away from *C. albicans* to *Candida* non-albicans species, was observed (Abi-Said 1997; Marr 2004). In the 2003 SENTRY antimicrobial surveillance programme, *C. albicans* occurred in 48.7% of all cases, *C. parapsilosis*, and *C. glabrata* both accounted for about 17% of the collected isolates, followed by *C. tropicalis* at 10.9% (Messer 2006).

The *Candida* species are normal flora of the human gastrointestinal tract, mucous membranes and skin. As much as 32-62% of healthy people carry the organisms in their oral cavity (Burford-Mason 1988; Hauman 1993), 48% in healthy infants mouth (Darwazeh 1995), and 43.7% in children (Rozkiewicz 2006). 17%

of medical students had the organism on their fingers (Clayton 1966), while carriage on health care workers' hands were as high as 59% (Brunetti 2008; Strausbaugh 1994; Yildirim 2007).

Candida organisms are eukaryotic. Cells are predominately unicellular, oval, and about 4-6μm in size. The fungal cell wall is composed three types of glycans: mannan, glucan and chitin (Masuoka 2004). The mannoproteins are located just outside the cell membrane. Followed by β-1,3- and β-1,6-glucan layers covalently bonded with chitin (Osumi 1998; Tronchin 1981). The outermost surface is layered with mannoproteins arranged in fribrillar structures (Cassone 1978; Shepherd 1987). These cell wall components are much pursued targets for the development of diagnostic tests and drug targets. They also aid in the understanding of *Candida* pathogenesis (Alexander 2002; Debono 1994; Georgopapadakou 2001; Kędzierska 2007; Poulain 2004). The plasma membrane has a phospholipid bilayer, with cylindrical ergosterol inserted in it for memebrane integrity (Odds 2003) This sterol and its synthetic pathways are targets of polyenes (e.g. amphotericin B), azoles (e.g. imidazoles and triazoles) and allymines (e.g. terbinafine) (Georgopapadakou 1994; Ghannoum 1999) . Genetically, *C. albicans* is diploid with eight chromosomes (Scherer 1990; van het Hoog 2007). It is

generally thought to be asexual, although mating locus was identified in 1999 (Hull 1999), however the significance of which is still debatable (Bennett 2009; Bougnoux 2008; Nielsen 2007). Morphologically, *C. albicans* are dimorphic with budding yeast cell phase, and pseudohyphae / hyphae formation. Such filamentation or mycelial formation had long believed to be a virulence factor in the invasion of host tissues through experiments on contact sensing and mutants construction (Lo 1997; Sherwood 1992).

In the laboratory, the *Candida* organisms appear as Gram-positive budding yeasts on Gram's staining. In clinical specimens, apart from yeast forms, pseudohyphae and hyphae can also be found. They grow well on most laboratory media. On blood agar, they appear as small, white and smooth colonies that look like staphylococci after overnight incubation. On prolonged incubation, 'foot-like' processes at the edge of the colony can be seen with species such as *C. albicans*. Germ-tube formation can be readily performed by suspending the colonies into horse serum and incubated at 37°C for 2 hours. The presence of germ-tube identifies the organism as *C albicans*. It needs to be cautioned that *C. tropicalis* also produces hyphae (with basal constriction) which closely resemble germ-tube (Potter 1991). This could result in mis-identification. Further identification of

various *Candida* species requires morphological identification by growing the organism on cornmeal agar under microaerophilic condition (Larone 2002; Warren 1999). Other methods of identification include carbohydrates assimilation test, substrate enzymatic tests, chromogenic agar test and polymerase chain reaction with single strand conformational polymorphism or restriction enzyme analysis (Heelan 1998; Houang 1997; Hui 2000; Maiwald 1994).

Speciation of *Candida* organism had recently gained much attention. It is now recognized that antifungal susceptibility result is related to the type of *Candida* species isolated (Pappas 2009; Pfaller 2005, 2007). *C. albicans* and *C. parapsilosis* are uniformly susceptible to azole agents, whereas *C. glabrata* and *C. krusei* both could harbor intrinsic resistance. *C. lusitaniae* and *C. guilliermondii* are both less susceptible to amphotericin B (Collin 1999). This has lead to the need of correct and timely speciation of *Candida* species from invasive diseases. It is not sufficient to simply classify them into *C. albicans* and non-albicans. Clinicians should not accept less than the best standard of care for their patients (Aliyu 2006; Denning 2003b; Schelenz 2009).

2.2 *Cryptococcus neoformans*

The *Cryptococcus neoformans* species complex is a group of yeasts characterized by a large extracellular capsule. It was first described in 1894 by Busse, who recovered the yeasts from a woman's tibia. In the same year, Sanfelice discovered it from peach juice (Barnett 2001; Lin 2006). This suggested a wide ecological niche for this organism. Although initially thought to be a single species, it was subsequently divided into 4 serotypes A, B, C, and D based on antigenic differences in the capsular polysaccharides (Belay 1996). Serotypes A and D are also known as *Cryptococcus neoformans var. neoformans*, serotype B and C as *Cryptococcus neoformans var. gattii.* The former has worldwide geographical distribution, whereas the latter was found mainly in the tropics and subtropical areas. The *neoformans* variant has its ecological niche in pigeon droppings and soil contaminated with it, while the *gattii* variant is associated with the *Eucalyptus camaldulensis* tree in Australia. Apart from ecological differences, the two varieties also differ clinically. Immunocompetent individuals are more at risk of *gattii* variant infection and have a poorer prognosis (Chen 2000; Mitchell DH 1995; Speed 1995). Recently taxonomists had advocated furthering dividing the species into 8 variants. However, there is no relevant clinical need for another change in

nomenclature (Kwon-Chung 2006).

As the organisms are ubiquitous in the environment, aerosolisation of contaminated soil followed by inhalation of the particles was thought to be the main route of infection. Using the Andersen air sampler, Bulmer had demonstrated that the pigeon droppings contaminated soil particles could be aerosolized to as small as 2μm, minute enough to be inhaled into distal respiratory tract (Bulmer 1990; Neilson 1977). The organisms probably then enter into a dormant state, and only reactivate and disseminate when the host response, such as T-cell immunity, is lowered (Lin 2006). The organisms exhibit neurotropism and seed to the central nervous system, resulting in meningoencephalitis (Lee 1996). It has been postulated that dopamine and epinephrine can be used by *Cryptococcus* in the production of melanin, which could be protective against phagocytosis and antifungal agents (Gómez 2003).

Cryptococcus neoformans is eukaryotic. Cells are unicellular, oval and about 5μm is size. The capsule can have a thickness the same size as the cell, if not bigger. The encapsulated yeast cells are readily visualized by India ink negative staining, in which the organisms appear as a budding yeast cells surrounded by bright halos. It is readily cultivable on most laboratory media. After overnight

incubation, the colonies are usually very tiny. Upon further incubation for 2 days, the colonies grow to a diameter of about 2 – 3 mm. Colonies are white and mucoid, with smooth edge and surface. In contrast to *C. albicans*, most isolates of *Cryptococcus neoformans* are haploid with 14 chromosomes, with defined sexual cycle involving two mating types: MATa and MATα (Loftus 2005). The α-strains itself is a virulence factor and is the more prevalent strain (Kwon-Chung 1992; Nielsen 2003). The organisms can undergo mating (between MATa and MATα) or same sex monokaryotic fruiting (Lin 2005). Either way, it undergo meiosis and forms basidiospores (< 2μm) that can be aerosolized and dispersed in nature (Wickes 1996).

Infections by this organism are generally considered as secondary to individual immunocompromisation, whereas person-to-person spread is largely unknown. In 2005, an outbreak erupted with the tropical strain *Cryptococcus neoformans var. gattii* in Vancouver Island, Canada, (Hoang 2004; Stephen 2002). This outbreak affected both men and animals. Subsequent analysis revealed that same sex mating of a hypervirulent strain had lead to an expansion of ecological niche and propagation of the outbreak (Fraser 2003, 2005; Kidd 2004).

2.3 *Pneumocystis jirovecii* (*Pneumocystis carinii*)

This organism was first observed in 1909 by Carlos Chagas in guinea pig lung specimens. However, he mistook it as trypanosome species and named it *Schizotrypanum cruzi* (Stringer 1996). Next year, the organisms were rediscovered by Antonio Carini in lungs of rats. In 1912, Delanoe considered the organism new protozoa, and designated it as *Pneumocystis carinii* in honour of Antonio Carini (de Souza 2005). For years, this organism was considered an animal protozoan with little clinical importance. In 1952, Otto Jiroveci described the organisms as a cause of pneumonia in neonates. Frenkel subsequently noticed that the *Pneumocystis* organisms recovered from humans and rats were different in physiology, pathology and host specificity. Thus he proposed that *Pneumocystis* organisms from different hosts should be considered as different species. Based on this, he suggested renaming the organism from human as *Pneumocystis jiroveci* (Frenkel 1976). After much debate, a consensus was finally reached in 2002 that the organism causing human disease is named *Pneumocystis jiroveci* (Stringer 2002). *Pneumocystis carinii* will be retained for species that cause infection in rats. In compliance with the International Code of Botanical Nomenclature, the name is further refined to *Pneumocystis jirovecii* (Redhead 2006). The commonly used

acronym PCP will be retained for Pneumocystis pneumonia (Stringer 2002).

Pneumocystis jirovecii are no ordinary fungi. Morphologically they resemble protozoa closely with both trophozoites and cysts stages. However, RNA sequencing results and the identification of fungus specific elongation factor provided the earliest clue that they are fungi rather than protozoa (Edman 1988; Ypma-Wong 1992).

The pneumocystis trophozoites are small (0.3μm), pleomorphic and tend to form clusters. This is the form usually identified in infected human lungs. The trophozoites are haploid. They can divide by binary fission or conjugate to form a diploid cell. The latter will undergo meiosis and nuclei division, resulting in 8 intracystic bodies and mature into cyst stage (de Souza 2005; Matsumoto 1984). While its ecological niche is still not well known, the organisms are thought to be ubiquitous and acquired through inhalation. Many people would have been exposed to this fungus at an early age (Peglow 1990). Its disease usually manifests as pneumonia. Extra-pulmonary infections are rare (Ng 1997). Much of the early literature concerned malnourished children after World War II (Russian 2001). Subsequently it was recognized as opportunistic pathogens in men suffering from

immunosuppressive diseases such as HIV, malignancies, use of steroids or cancer chemotherapeutic agents (Sepkowitz 1993).

Chapter 3: Diseases

3.1 Candida

3.1.1 Candida blood stream infections

Candidaemia refers to the isolation of *Candida* organism from blood cultures. Surveillance studies had indicated a secular rising incidence of candidaemia in hospitals, especially in intensive care units (Edgeworth 1999; McGowan 1975, 1985; Pittet 1995; Schaberg 1991). In the United States, the NNIS and SCOPE data revealed that 9% to 11.5% of bloodstream infections were due to *Candida*. It only ranked behind coagulase-negative *Staphylococcus* species, *Staphylococcus aureus* and enterococci in these studies (Edmond 1999; Richards 1999, 2000; Wisplinghoff 2004). This represented a tremendous increase compared to the 3.8% (the eighth rank) reported in 1953 (Hobson 2003; McGowan 1975). Incidences of candidaemia vary between countries and states. In 2003, Kibbler *et al* reported an incidence of 3 per 100,000 bed days in England and Wales (Kibbler 2003). In Scotland, Odds *et al* reported an incidence of 5.9 per 100,000 bed days (Odds 2007). Using population-based surveys, the annual incidences were 6 and 24 per 100,000 population in Iowa and Baltimore respectively (Diekema 2002; Hajjeh 2004). European surveillance reports indicate mean incidences of 3.1 and 4.4 per 100,000 patient days in Germany/Austria and Italy respectively (Tortorano 2004).

Differences in patient characteristics, risk profiles, and medical practices would probably account for the differences (Pfaller 2007). Among these infected patients, risk factors such as prior usage of broad spectrum antibiotics and/or immunosuppressant, presence of vascular catheters, neutropenia, recent gastrointestinal surgery and prolonged ICU stay were all well validated (Alonso-Valle 2003; Bross 1989; Dimopoulos 2007; Holley 2009; Nolla-Salas 1997; Schelenz 2003; Vardakas 2009;).

As these infections occur in patients who are particularly vulnerable, mortality is high. In the adult patients, the crude mortality rates ranges from 34% to 79%. The attributable mortality rate is 21.5% to 43% (Ellis 2001; Fraser 1992; Macpharil 2002; Marriott 2009; Meunier-Carpentier 1981; Nolla-Salas 1997; Wey 1988). However, with the advent of newer antifungal agents, prudent antibiotic use, recognition and removal of risk factors, there are now data which suggest that the mortality might be lowered (Chen 2003) In paediatric patients, overall mortalities range from 15.8% to 29% (Pappas 2003; Stamos 1995; Zaoutis 2005). Particularly high rates of 39% to 76% were reported from intensive care units and those with congenital heart diseases (Kuzucu 2008; Pasqualotto 2005; San Miguel 2006). While children with burns, but otherwise healthy, had low mortality rates of 5.9%

(Sheridan 1995).

Among the various species of Candida, isolation of *C. albicans* had shown a decreasing trend with time (Trick 2002). In an analysis of more than 200,000 clinical isolates collected from 40 countries, isolation of *C. albicans* had fallen from 70% in 1997 to 63.2% in 2005 (Pfaller 2005, 2007). Isolation of other species, notably *C. glabrata*, *C. tropicalis* and *C. parapsilosis* had slowly increased over this time period (Hachem 2008). These 'top four' organisms accounted for 91% of all clinically significant *Candida* isolates. The increasing prevalence of *Candida* non-albicans was more prominent in the ICU settings. Numerous studies have documented an increase in isolation of *C. glabrata* and *C. krusei*, which possess intrinsic resistance to fluconazole (Bassetti 2006; Davis 2007; Hope 2002; Ruan 2008a, 2008b; Shorr 2007). One may postulate that this is due to the widespread use of fluconazole in both treatment and prophylaxis. The emergence of these resistant species can lead to a downward spiral of antifungal resistance, because it drives the empirical use of even more potent antifungals such as the echinocandins (Mensa 2008; Pappas 2009). *C. parapsilosis*, one of the 'top-four' candidaemic organisms, was noted to have elevated caspofungin and micafungin MIC levels in some of the isolates (Ghannoum 2009; Moudgal 2005; Pfaller 2008a, 2008b).

Thus, prudent antifungal use is of utmost importance.

Candida organisms are part of the normal flora. Thus candidaemia is mainly an endogenous infection. When the skin or mucous membrane barrier is disrupted, *Candida* will have the opportunity to invade (Jarvis 1996). Certain chemotherapeutic agents, such as high dose cytarabine (Ara-C), are associated with severe mucositis, resulting in higher risk of developing candidaemia (Koh 2004; Sallah 2001). Gastrointestinal surgery was also associated with candidaemia in critically ill patients (Shivaprakasha 2007; Vardakas 2009). This has lead some to advocate the use prophylactic fluconazole therapy in gastrointestinal surgery (Shan 2006). Percutaneously inserted vascular catheters, especially central venous catheters are associated with high risk of candida bloodstream infections (Cauda 2009). Most guidelines recommend the removal of vascular catheters in the event of candidaemia (Denning 2003b; Wolf 2008; Pappas 2009). However, recent analysis called upon a review on this routine practice, suggesting a lack of improvement in clinical outcomes with the systematic removal of catheters (Pasqualotto 2008; Rodriguez 2007). These factors are probably stages in a continuum of risks. For example, multiple antibacterial exposures would lead to an imbalance of normal flora, resulting in overgrowth of *Candida* species (Huang

2003). The increased skin carriage might have lead to a higher chance of vascular catheter colonization and subsequent bloodstream infections (Fridkin 1996).

Exogenous infections are fortunately uncommon. Several outbreaks have occurred and linked to: cross-contamination from hands of healthcare workers; contaminated substances being administered intravascularly (e.g. parenteral nutrition, anesthetic agents, or medications); contaminated devices such as intravascular pressure monitoring devices or vacuum pump; practice of retrograde medication administration; or environmental contamination. If specific point source can be identified, improvement in clinical practices will be sufficient to control the outbreak. Even if the point source cannot be identified with confidence, reinforcement in infection control practices would help (Bennet 1995; Burnie 1985; Chowdhary 2003; DiazGranados 2008; Dizbay 2008; Koc 2002; Masala 2003; Moro 1990, Pfaller 1995b; Sherertz 1992; Solomon 1984; Weems 1992). Several outbreaks had documented the relationship between *C. parapsilosis* and central vascular catheter / parenteral nutrition use (DiazGranados 2008; Dizbay 2008; Levin 1998; Plouffe 1977; Solomon 1986; Weems 1987). This organism is capable of producing biofilm and adhering onto acrylic surfaces, peritoneal catheters, and prosthetic heart valves (Diekema 1997; Johnston 1994; Kaitwatcharachai 2001;

Kuhn 2002, 2004). Together with its ability to grow in sugar rich solution, it can proliferate in total parenteral nutrition, adhere to vascular catheters, and result in blood stream infections (Almirante 2006; Brito 2006; Fridkin 1996; Pfaller 1995a; Tumbarello 2007;).

Clinical manifestations of candidaemia are often non-specific. Typically the patient belongs to one of the high risk groups: e.g. neutropenic, leukaemic, prolonged stay in ICU, post-gastrointestinal surgery, on total parenteral nutrition / cytotoxic drugs / corticosteroids or multiple broad spectrum antibiotics. Many a times they present with fever or sepsis with no specific origin. Occasionally a macronodular skin rash may appear. If the disease disseminate to other organs (e.g. endophthalmitis or arthritis), relevant signs and symptoms may develop. However, dissemination to deep seated organs (e.g. liver or spleen) may not be readily detectable unless radiological investigation is performed. Conventional blood cultures, although not very sensitive, remain the main workup tool in everyday practice (Méan 2008, Pappas 2006).

3.1.2 Disseminated Candida infections

Disseminated *Candida* infections refer to the situation where the *Candida* organisms seeded to other anatomical sites. Hepatosplenic candidiasis occurs more commonly in neutropenic patients. Abdominal pain, hepatomegaly and raised alkaline phosphatase may be found. The origin of the *Candida* organism is probably from the gastrointestinal tract via mucosal breaks or translocation. The typical "bulls-eye" / halo-sign appearances on CT scan of the organs may not be obvious until the recovery of neutropenia, but early signs of multiple small space occupying lesions may be seen. To establish the diagnosis, culturing of the lesion is desirable, but not always possible in the critically ill patients. However, even when biopsy is available, the culture results are often negative.

The *Candida* organism can also spread haematogenously to other organs such as the eyes, seeding onto the choroids and retina resulting in chorioretinitis, vitritis, or endophthalmitis (Sallam 2006). This condition is sight-threatening and could be bilateral. Since patients are often critically ill, and may not be able to articulate any symptoms such as blurred vision or ocular pain, fundoscopic examination is advisable in all patients with candidaemia. Early consultation with the

ophthalmologist is important as these patients may require intra-vitreal antifungals and vitrectomy (Khan 2007; Lamaris 2008).

Involvement of other anatomical sites is relatively uncommon, but has been reported especially in immunocompromised patients (Crum-Cianflone 2008). *Candida* myositis, presenting with muscle pain, fever and rash in candidaemic patients should prompt suspicion of the diagnosis (Jarwoski 1978). Microabscesses are formed in the affected muscle; radiological investigation is helpful to delineate the extent of involvement and to guide biopsy for culture (Kressel 1978; Schwartz 2008). Candida species such as *C. albicans*, *C. tropicalis*, and *C. krusei* have been reported as the causative agents (Arena 1981; Diggs 1976; Fornadley 1990; Schwartz 2008; Tsai 2006). *Candida* osteomyelitis can present acutely together with the episode of candidaemia, or months after the bloodstream infection episodes (Miller 2001). Common symptoms are fever and localized pain. In infants and children, mainly the long bones are involved. In adults, the vertebrae are the commonest site of infection (Arias 2004; Shaikh 2005). Blood cultures should be performed, although they are positive in only about 50% of cases. Radiological delineation with the use of MRI would be the method choice in bone infections (Lew 1997). Biopsy and culture of the infected bone would provide

definite proof of the infection. *C. albicans* constitute the majority of reported cases. Other species such as *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, *C. kefyr*, *C. guilliermondii* and *C. dubliniensis* have all been reported (Arias 2004; Miller 2001; Wellinghausen 2009). *Candida* endocarditis mainly occurs in intravenous drug users and patients with prosthetic valves. In neonates and children, congenital cardiac malformations are additional risk factors (Bacak 2006; Lye 2005; Millar 2005). *Candida* pericarditis is rare and occurs mainly after cardiothoracic surgery and occasionally in immunocompromised patients (Kaufman 1988; Neughebauer 2002; Rabinovici 1997; Schrank 1995;). *Candida* myocarditis was rarely reported in the literature (Einarsdottir 2002; Hall 1986). *Candida* pneumonia is difficult to ascertain in clinical practice. *Candida* colonization of the respiratory tract is common in immunocompromised patients. The diagnosis of *Candida* pneumonia is therefore notoriously difficult to differentiate from mere colonisation (Schwesinger 2005). Most patients at risk are critically ill, requiring ventilator support, which precludes aggressive diagnostic measures such as transbronchial biopsy or open lung biopsy. Few reported cases have biopsy proof (Chen 2001; Petrocheilou-Paschou 2002). Most authors opted for bronchoalveolar lavage cultures and/or semi-quantitative cultures as a guide for confirming the diagnosis (Delisle 2008; Kalkanci 2005; Wood 2006). However, the interpretation of these

specimens has always been problematic. Immediate post-mortem analysis has suggested a poor correlation between culture results and true infections (El-Ebiary 1997). Clinicians were divided in their opinions and practices (Azoulay 2004). Therefore, until a better diagnostic method is available, *Candida* pneumonia remains a disease difficult to confirm. *Candida* meningitis is associated with neurosurgical intervention and the use of intraventricular catheters or lumbar drains (Sánchez–Portocarrero 2000). Micro-abcsesses are formed at the junction of grey-white matter. Histology may show hyphae invading into the brain parenchyma. This rare disease is often difficult to diagnose antemortem (Parker 1978; Pendlebury 1989). *Candida* peritonitis is often related to perforation of the gastrointestinal tract, especially the upper part; or recent surgery of the abdominal cavity (Blot 2007). In peritoneal dialysis associated fungal peritonitis, removal of the peritoneal catheter is often required to ensure a cure of the disease (Prasad 2005; Raaijmakers 2007).

3.2 Cryptococcus neoformans

Cryptococcus neoformans infections are uncommon and occur sporadically. Reported cases are usually associated with some from of immunosuppression such

as diabetes mellitus, autoimmune disease, or corticosteroid use. With the HIV/AIDS epidemic, more cases were observed, which can be categorized broadly as meningoencephalitis or extraneural diseases (Mitchell TG 1995).

3.2.1 Meningoencephalitis

Cryptococcal meningoencephalitis is typically insidious in onset, presenting with headache, fever, mild meningeal irritation, confusion and rarely psychiatric disturbance over a few weeks. In HIV/AIDS patients, concurrent foci of infection may be present (e.g. cryptococcaemia or pulmonary disease). Defects in T-cell immunity, and AIDS with CD4 count of < 100/ μL are the main risk factors (Mitchell TG 1995). As the basal meninges are preferentially affected, imaging may show meningeal enhancement (Subramanian 2005). Occasionally cryptococcoma may be seen (Offiah 2006). Cerebral edema and hydrocephalus are occasionally observed also (Chan 1989). Cerebrospinal fluid analysis shows predominance of lymphocytes, and mildly abnormal glucose and protein. The organisms can be observed as encapsulated yeasts, which appear as a halo on India ink preparations of CSF. Cryptococcal antigen detection is a useful diagnostic test on CSF or serum, and is readily available in commercial kit form. Although

cryptococcal meningitis is one of the AIDS defining diagnoses and occurs commonly in immunocompromised patients, it is now realised that as much as 22% of patients with cryptococcal meningitis have no identifiable risk factor (Pappas 2001).

3.2.2 Extraneural Cryptococcal Diseases

Extraneural diseases are commoner in HIV/AIDS patients, who also suffer from higher rates of relapse and persistent bloodstream infection. Pulmonary cryptococcal infections can present as acute fulminating pneumonia in the immunocompromised, or more indolently with cough, weight loss and dyspnoea in the immunocompetent host. Chest x-ray findings range from solitary pulmonary nodules to diffuse lung infiltrates. Confirmation of the diagnosis would require lung biopsy for culture and histology. Serum cryptococcal antigen may be useful, but may remain negative for confined pulmonary disease (Campbell 1966; Jarvis 2008). Cutaneous Cryptococcus could represent primary inoculation in immunocompetent hosts or disseminated cryptococcal diseases in the compromised host (Christianson 2003; Lacaz 2002). Clinical appearances are highly variable and ranging from nodular, papular, ulcer, abscess, cellulitis, herpetiformis-like, to

basal-cell carcinoma-like (Borton 1984; Murakawa 1995, 1996; Singh 1994). This highlights the importance of culture and histological confirmation in patients at high risk. Cryptococcoccus infection in the prostate is usually an incidental histological finding after resection of prostate (Braman 1981). Cryptococcaemia after transurethral resection of prostate (TURP) has been reported, and the prostate has been postulated as a possible niche in patients with relapses (Allen 1982; Larsen 1989; Staib 1990). However, this is likely of minor clinical relevance since HIV/AIDS patients with cryptococcal meningitis and low CD4 count will be maintained on chronic fluconazole therapy, which has good penetration into prostatic tissue (Finley 1995; Luzzati 1998; Mitchell 1995). Cryptococcal infections of the eye are sequelae of meningeal infections. This presents with ocular palsies, papilloedema or visual loss. Rex *et al* described two clinical types: one with rapid vision deterioration within hours and associated with optic neuritis; another type with slow visual loss of over weeks to months probably associated with background raised intracranial pressure (Rex 1993). It occurs in about 3% of HIV/AIDS patients and carries extremely poor prognosis in terms of visual recovery (Torres 1999).

3.3 Pneumocystis jirovecii

3.3.1 Pneumonia

Pneumonia is the main presentation of *Pneumocystis jirovecii* infection. It is generally believed to be acquired via inhalation or airborne route. Gradual onset of shortness of breath, chest pain, non-productive cough and desaturation on exertion are the main symptoms (Kales 1987). This typically occurs in HIV/AIDS patient whose CD4 count is < 200/μL (Lu 2008). Other immunocompromised patients such as those on cytotoxic therapy and transplant recipients are also at risk (Vento 2008). However, these groups of patients usually present with fulminant pneumonia and respiratory failure (Bollee 2007; Yale 1996). Diffuse interstitial or alveolar infiltrates are observed on chest x-ray, while CT scan may reveal patchy or nodular ground-glass appearance (DeLorenzo 1987; Hartman 1994). Diagnosis is confirmed by demonstration of the organism in induced sputum, bronchoalveolar lavage, transbronchial biopsy. With the usage of HAART, as well as primary and secondary prophylaxis for pneumocystis infection in HIV infected individuals, and probably in selected groups of immunocompromised patients, it is envisaged that the incidence of *Pneumocystis jirovecii* infections may further decrease (Green 2007, Sullivan 2001).

3.3.2 Extrapulmonary infections

Extrapulmonary *Pneumocystis* infections are rare diseases. The use of aerosolized pentamidine in HIV/AIDS patients for PCP prophylaxis appeared to coincide with an increase in extrapulmonary diseases. Therefore there were concerns that inhalational pentamidine may not protect against systemic infections. As the number of cases is still exceedingly small, this remains controversial. Nonetheless, for clinicians caring for HIV/AIDS patients, possibility of atypical infection should be born in mind. In HIV positive patients, the involved sites are the ears (presenting with hearing loss and aural polyp) and the eyes (ranging from asymptomatic choroiditis to visual loss). In HIV negative patients, widespread involvement of multiple organs such as liver, spleen and kidney were reported (Ng 1997).

Chapter 4:
Diagnostic Methods

4.1 Candidaemia

4.1.1 Culture-based methods

Recovery of the organism by culture remains the standard proof of infection. This is usually achieved by conventional blood cultures. In recent years, blood culture systems have evolved from manual biphasic culture method to fully automated system with continuous monitoring, as well as lysis-centrifugation method (Berenguer 1993; Reimer 1997). Their advantages include assessment of time-to-positivity and detection of intracellular organisms. However, culture sensitivity can be as low as 25%, especially with hepatosplenic candidiasis where the organisms are likely to be encased (Jones 1990; Reiss 1993; Telenti 1989; Thaler 1988). In these circumstances, biopsy of the infected anatomical sites for culture and histology would provide definitive proof. However, these are not always possible in critically ill patients. Although culture is insensitive, they are still extremely useful as positive growth provide the organism for identification, susceptibility testing and occasionally outbreak investigation.

4.1.2 Non-culture based methods

4.1.2.1 Antibody detection

The detection of antibodies against *Candida* organism is hampered by the fact that healthy individuals also harbor antibodies against these normal flora. To date, no antibody detection method had been reliably put into routine clinical service.

4.1.2.2 Antigen detection

Detection of fungal cell wall antigens has been explored as a diagnostic tool. Mannan, a fungal cell wall component, has been used for many years. Unfortunately, the rapid clearance of the anitgenaemia from serum, and the use of insensitive methodologies such as radioimmunoassay and polyclonal antibodies, has resulted in a lack of sensitivity. Even with the use of newer immunodiagnostic technique, the sensitivities is only about 40% to 73% (Sendid 1999; Yera 2001). Similarly, other antigens such as enolase did not gain wide acceptance (de Repentigny 1992; Matthews 1996; Walsh 1991).

Currently there is renewed interest in antigenaemia testing with the use of β-D-glucan. This cell wall antigen is present in a large range of fungal organisms,

except *Cryptococcus* and *Zygomycetes*. Extremely low levels of antigen (in terms of pictogram) can be detected, thus making it a very sensitive test (Odabasi 2004; Stevens 2002). While the sensitivity is greatly enhanced, it is hampered by false positivity from environmental fungi. The extreme sensitivity of the test also requires it to be done with special precaution, such as handling inside a safety cabinet, and using pyogen-free reagents and containers. None the less, promising strategies such as serial measurement of β-D-glucan to monitor treatment response, and the institution of preemptive therapy in high risk groups had been advocated (Pazos 2006; Senn 2008). Further understanding of the kinetics of β-D-glucan in the human body would enhance its clinical usefulness.

4.1.2.3 Metabolite detection

Ability to detect unique metabolites from the pathogenic organism is an attractive diagnostic option. In *Candida*, the 5-carbon sugar D-arabinitol is the commonly explored. Most *Candida* cells produced D-arabinitol, while human cells produce both D- and L-arabinitol, it was conceived that measurement of the relative differences could be a useful diagnostic tool (Bernard 1981; Christensson 1999). The test can be performed on minute quantity of samples such as serum from a heel-stab of neonates, making it attractive for use in children where blood taking can

be difficult. The main difficulty lies in the diffentiation of the enantiomers, which is technically difficult and requires costly instruments (Eisen 2002; Larsson 1994). Thus widespread adoption into routine laboratories is difficult.

4.1.2.4 Detection of nucleic acids

Molecular technology allows specific nucleic acids to be detected. Highly conserved ribosomal genes are the usual targets. These genes also carry variable domains that allow further identification of specific fungal species. Nested PCR may be required to enhance the sensitivity of the molecular test (Yeo 2002). The diagnostic sensitivity and specificity were reported to be high (Ahmad 2002; McMullan 2008). As many laboratories are now equipped with the infrastructure for molecular diagnosis, it is foreseeable that molecular fungal diagnostics may be put into routine clinical service when validation and adaptation of such technique become easier (Alexander 2002).

Many of these non-culture based diagnostic methods still need to be vigorously assessed in large clinical trials. Importantly, specific patient populations such as neonates and children need to be targeted before the test performance can be comprehensively assessed (Einsele 2008; Wheat 2006).

4.2 Cryptococcocus neoformans

In meningoencephalitis, the mainstay of diagnosis is the examination of cerebrospinal fluid. India ink is a classical technique to reveal the large halo of yeasts' capsules. For the inexperienced, confusion may arise when lymphocytes are seen, as these cells also appear as a bright spot. However, the lack of budding and the its round-shape should have alerted the microscopist. About half of non-HIV infected patients, and a quarter of HIV-infected patients will have a negative India ink result (Dismukes 1987). In these situations, detection of the cryptococcal polysaccharide antigen in CSF is a useful diagnostic test, especially when the clinical picture and CSF findings are compatible. Alternatively, serum can also be tested for cryptococcal antigen (Mitchell TG 1995). On the other hand, temporal monitoring had not shown to be a useful prognostic marker (Antinori 2005; Powderly 1994). Finally, culture of CSF and/or blood and other relevant sites will provide definite proof of the disease, and is almost always positive in HIV-infected patients (Gal 1987). Radiological evaluation is important, as cryptococcal meningoencephalitis can present with raised intracranial pressure and hydrocephalus. Moreover, other space occupying lesions such as lypmphomas can present with

neurological symptoms, or co-exist with cryptococcal infections.

4.3 Pneumocystis jirovecii

This organism cannot be cultured routinely *in vitro*. So the demonstration of the cyst and/or trophozoits is mainly by special staining of patients' samples. Toluidine blue O stain, Grocott's silver stain or Giemsa stain can be used on respiratory secretion specimens. Bronchoalveolar lavage is the preferred specimen. Sensitivity can be as high as 90% in HIV-infected individuals (Lu 2008). In situations where the patients cannot tolerate bronchoscopy, induced sputum may be used, and has been reported to have sensitivities of 74% (Ng 1989). On microscopy, the organisms appear collapsed and clump together against a background of scanty white cells. Molecular diagnostic with the use of PCR is now under active exploration (Fischer 2001; Larsen 2002; Torres 2000). In general, PCR appears to be more sensitive, but less specific. However, this may be due to early institution of treatment which decreased the organisms load, resulting in a reduction of sensitivity (Larsen 2004).

Chapter 5: Treatments

Development and discovery of antifungal agents had always lagged behind antibacterials. While Alexander Fleming discovered penicillin in 1929, it was not until 1949 that the first antifungal nystatin was discovered (Fleming 1929; Hazen 1950). In 1953, amphotericin was discovered from screening of *Streptomycetes* species, its subsequent purification yielded the first useful systemic antifungal agent amphotericin B (Dutcher 1968).

5.1 Polyenes

These are large molecules with a conjugated double bond system and a macrolide ring, resulting in a cyclic lactone. Opposite to the double bonds are a number of hydroxyls of 1,3-relationship. The polyenes are amphiphathic, with a lipophilic polyene region and a lipophobic polyol region. These agents bind to the sterols of fungal cell membranes, resulting in disruption and subsequent lysis. The first polyene, nystatin, proved to be a potent sclerosing agent when injected intravenously. On oral administration, it is not absorbed by the gastrointestinal tract. Thus, it has not developed into a useful agent for the treatment of invasive fungal infections (Kidwai 2003).

Amphotericin, after its discovery in 1953, was subsequently divided into two types: amphotericin A and amphotericin B. It was soon noted that amphotericin B is much more potent than it's A counterpart, resulting in the purification and manufacturing of amphotericin B for clinical use (Aszalos 1985; McNall 1957). Due to its higher affinity for fungal ergosterol rather than cholesterol in mammalian cell membrane, it is less toxic and much more tolerable than nystatin. It has activity against most yeast and molds. Large scale industrial production was possible by mixing amphotericin B with sodium deoxycholate forming a complex that can be reconstituted in dextrose solution for intravenous usage. However, it is still significantly nephrotoxic, with a decrease in glomerular filtration rate, renal wasting of potassium and magnesium, and renal tubular acidosis (Branch 1988; Bates 2001; Harbarth 2001; Sawaya 1995). In fact, Symmers described "amphotericin pharmacophobia" in which delayed antifungal therapy due to fear of its toxicity (Symmers 1973). Nephrotoxicities are also related to the dose administered (>0.5mg/kg) and concomitant usage of other nephrotoxic agents. This could lead to an inadequate amphotericin B dose being prescribed (de Marie 1994; Fisher 1989). One measure to minimize toxicity is salt loading. Giving 1000mL saline intravenously before amphotericin B appears to provide a renal protective effect (Girmenia 2005; Llanos 1991). In the quest for a better antifungal

drug, in 1983, Lopez-Berestein *et al* experimented on liposome-encapsulated amphotericin B in murine candidiasis, and conducted the first clinical trial in 1985. The liposomal formulation was observed to have much reduced toxicity, probably due to increased affinity for fungal cell membrane (Lopez-Berestein 1983, 1985, 1989). To date, several formulations of lipid-based amphotericin B are licensed: (1) amphotericin B lipid complex (ABLC) which are ribbon-like complexes; (2) amphotericin B colloidal disperision (ABCD) which are disc-like complexes of cholesteryl sulfate and amphotericin B; and (3) liposomal amphotericin B (LAMB) which consists of spherical unilamellar liposomes (de Marie 1994; Dupont 2002). Manual emulsification of amphotericin B into 20% intralipid by vigorous agitation has been advocated (Chavanet 1992; Shadkhan 1997). However, precipitation has been reported, and conflicting data on safety, efficacy and stability precludes general recommendation of this mixture (Sievers 1996; Walker 1998). The lipid-based amphotericin Bs are not the same (Adler-Moore 2008; de Marie 1994; Dupont 2002). ABLC has a high volume of distribution (131 L/kg) when compared to ABCD (4.3 L/kg) and LAMB (0.11 L/kg), resulting in a lower serum concentration. LAMB has a higher C_{max} (83 mg/L) and AUC (555 mg.h/L) when compared to ABLC (1.7 and 5.9 respectively) and ABCD (3.1 and 43 respectively). ABCD has more infusion related side-effects (Bowden 2002; White 1998). Thus these agents

should not be interchanged without careful consideration of the pharmacokinetics and side effect profile (Pappas 2009).

5.2 Azoles.

The azoles were developed in the 1970s. The initial members are the imidazoles such as miconazole and ketoconazole. They act by inhibiting the cytochrome P450 dependent C-14 demethylase, therefore inhibiting the conversion of lanosterol to ergosterol in fungal membrane synthesis. In the 1990s, the armamentarium was further expanded with the development of the triazoles: fluconazole, itraconazole, voriconazole, posaconazole and more preparations are under development (Pasqualotto 2008). All azoles are cytochrome P450 inhibitors, thus their use must be considered carefully with regard to local genetic polymorphism and drug-drug interaction. Hepatotoxicity was sometimes observed, with resultant increase in transaminases or even fulminant liver failure (Gearhart 1994; Song 2005). Thus, monitoring of liver functions is advisable. Another problem with azoles is that *C. glabrata* has reduced susceptibility (susceptible but dose dependent) and *C. krusei* has intrinsic resistance against the azoles.

Fluconazole is one of the most commonly used triazoles. It has high (>90%) bioavailability, reasonable penetration into the CNS (>50%), and is generally well tolerated. It has a long half life of 24 hours, thus allowing once daily dosing regimen. It has good activity against *Candida* and *Cryptococcus* organisms (except *C. glabrata* and *C. krusei*) but no activity against moulds.

Itraconazole, on the other hand, has activity against *Aspergillus* species, and has been a mainstay of treatment for these infections. Experience on its use in invasive candidiasis is limited. Bioavailability of itraconazole is variable and dependent on the formulation. The capsule is only 55% bioavailable, while the solution form is 80% bioavailable (Prentice 2005; Willems 2001). Thus the two oral formulations cannot be interchanged directly.

Voriconazole is active against yeasts and moulds (except *Zygomycetes*). It has good bioavailability of approximately 90%, with CSF to serum ratios ranging from 0.22 to 1.0 (Lutsar 2003). It is extensively metabolized by cytochrome P450 enzymes CYP2C19, 2C9 and 3A4. Since genetic polymorphism occurs in 15% to 20 % Asian populations, the "slow metabolisers" exhibit much increased serum levels and are prone to a higher risk of hepatotoxicity (Ikeda 2004). In hepatic

insufficiency such as status Child-Pugh Classes A and B, a dose reduction of 50% is recommended (Boucher 2004). When it is used for prophylaxis, breakthrough infections with zygomycosis had been reported (Imhof 2004; Kontoyiannis 2005).

Posaconazole is newly licensed. It has good bioavailability of >90%, and a prolonged half-life of 35 hours (Ezzet 2005; Krieter 2004). It has extended activity against yeasts and moulds, including zygomycetes (Rachwalski 2008).

5.3 5-Fluorocytosine

5-fluorocytosince (5-FC) was synthesized in 1957 as an anti-tumour agent. However, it exhibited effects against *Candida* and *Cryptococcus*. The drug itself has no antifungal activity. After being taken up by the fungal cell, it is deaminated into 5-fluorouracil, which subsequently interferes with DNA and protein synthesis (Vermes 2000a). Resistance via mutation has been reported, thus restricting use of 5-FC as monotherapy (Normark 1972; Polak 1975). It is used together with amphotericin B as combination therapy. It bioavailability is nearly 90%, and it is eliminated mainly via the renal route with a plasma half-life of 4hr. Most significant side effects are haematological suppression (leucopenia,

thrombocytopenia) and hepatotoxicity (Vermes 2000b, 2000c).

5.4 Eichinocandins.

The echinocandins are novel antifungal agents that selectively target fungal cell wall β-(1,3)-D-glucan synthesis. This target site enables the agent to be selectively toxic, resulting in good tolerability and few side effects when compared with azoles and polyenes (Denning 2003a). Currently there are three echinocandins in use: caspofungin, micafungin and anidulafungin. Their oral bioavailability is low, necessitating intravenous administration. Dose adjustment is not required for renal insufficiency. In the event of hepatic insufficiency, caspofungin dose adjustment is required. They are active against *Candida* and *Aspergillus*. Due to broad spectrum of coverage and selective toxicity, the echinocandins are now the preferred choice for empirical antifungal treatment. It allows treatment to be started promptly before speciation of *Candida* is known. De-escalation of therapy to oral azoles can be used subsequently (Hof 2008; Lichtenstern 2008; Ruping 2008).

PART II: ORIGINAL STUDIES

Chapter 6:
The invasive diseases

6.1 Background and Objectives

Invasive fungal infections have gained renewed interest in recent years. Contributory factors include the emergence of HIV/AIDS infections, extensive use of immunosuppressant for various disease entities, and the use of invasive procedures. Many studies had been done in various countries to assess the impact of these diseases. Few reports had been done to understand the epidemiology in areas with low HIV/AIDS prevalence.

Hong Kong has a low prevalence of HIV/AIDS. Unlinked anonymous surveillance has been conducted by the Government Department of Health since the 1990s. The overall prevalence of HIV in the adult population is <0.1% (WHO 2000). In this regard, it can be hypothesized that invasive fungal infections may have a lower incidence in our locality.

On the other hand, 95% of our populations are ethnic Chinese. Another spectrum of medical illnesses is more prevalent in this ethnic group, for example nasopharyngeal carcinoma (NPC) and and systemic lupus erythematosus (SLE) (Chan 2002; Lau 2006).

In order to understand infections by yeasts organisms in this setting, a review of the *Cryptococcus* infections, *Pneumocystis jirovecii* infections, and candidaemia is presented.

6.2 Study One: *Cryptococcus neoformans* infections in a setting of low HIV prevalence.

6.2.1 Abstract

Cryptococcus neoformans is an encapsulated yeast capable of producing life-threatening meningitis and disseminated infections. Immunocompromised patients such as those with HIV infections are particularly at risk. In a locality where the HIV prevalence is low, the disease is often not suspected. This could lead to a delay in diagnosis and institution of antifungal treatment. Thus, we aimed to analyse this disease in our locality. A retrospective review was conducted on all patients' records with microbiological confirmation of *Cryptococcus neoformans* infection at the Prince of Wales Hospital during the period 1993 to 2008. A total of 37 non-duplicate patients were identified during this period. Twenty-seven had meningitis, 6 had blood stream infections, 2 had pneumonia, 1 retinitis and 1 CAPD-associated peritonitis. In 5 patients, the diagnosis was not diagnosed ante-mortem. Crude mortality was 43%. Rate of decline of CSF antigen titres did not correlate with serum antigen titres. As the disease appeared to be uncommon in our locality, a high index of suspicion is required.

6.2.2 Method

Patients and case definitions

A retrospective case analysis was conducted to review *Cryptococcus neoformans* infections from 1993 - 2008. Cases were identified from the laboratory log of the Microbiology Department of the Chinese University of Hong Kong. The laboratory provides diagnostic and consultative services to the Prince of Wales Hospital (a university affiliated tertiary referral hospital) and the Northern District Hospital (since 2002). Laboratory results were archived electronically by in-house software. Cryptococcal infection was defined by positive India ink results from cerebrospinal fluid, isolation of the organism from cerebrospinal fluid or any sterile body sites, together with compatible features of clinical infections. Central nervous system involvement was considered compatible when fever of $\geq 38.5^{\circ}C$, altered mental state or features of raised intracranial pressure were present. Raised intracranial pressure was defined as pressure of $\geq$20cm H_2O. Pulmonary infection was considered compatible when fever and radiological changes were present. Other clinical infections were considered on a case by case basis. Response was defined as clinical improvement with serological or microbiological evidence, and completion of antifungal treatment (as decided by the responsible clinicians). Relapse was defined as recurrence of clinical

signs/symptoms with microbiological evidence after antifungal treatments were completed (inclusive of induction and consolidation therapy in HIV seropositive patients) Clinical outcome was assessed on the date of death, last follow up or on Dec 31, 2008, whichever is later. Demographical, clinical and laboratory data was recorded on a standard log and reviewed. All data access was done in accordance to prevailing local regulations to ensure patient confidentiality.

Microbiological investigations

Microbiological diagnosis of *Cryptococcus neoformans* was confirmed by microscopic examination of the clinical specimens with Gram's stain and India ink stain (on CSF). This was followed by culture on blood agar, chocolate agar and Sabouraud agar aerobically for 2 days before discarding as negative. CSF with an abnormal cell count (defined as > 5 WBCs/mm^3 in adults, > 30 WBCs/mm^3 in neonates) or with deranged biochemical parameters is kept for prolonged culture for 5 days. Cryptococcal antigen testing or monitoring by latex agglutination (CALAS, Meridian, Connecticut, USA) was performed on the CSF and/or serum upon request from the referring clinicians and/or microbiologists. A titre of $\geq$ 1:8 was considered positive. When relevant, tissue cultures and blood cultures were taken as well. From 1993-1997, the laboratory used Bactec 660 blood culture system (Becton Dickinson, Sparks, MD);

frrom 1998 – 2008, BacT/Alert system (Organon Teknika Corp., Durham, NC) was used. All suspicious organism growths were confirmed by Gram's staining, carbohydrate utilization (API 20C AUX or 32C AUX, bioMerieux, Hazelwood, MO.; Vitek 2, bioMérieux, Marcy l'Etoile, France) and morphological appearance on cornmeal agar.

Statistical analysis

Categorical variables were analysed by chi-square test or Fisher's exact test. Continuous variables were analysed by Student's t-test. Rate of decline of cryptococcal antigen was assessed by the slope of linear regression of $\log_2$ of antigen titres against time. The correlation between the rate of decline of CSF and serum cryptococcal antigen titres was assessed by Pearson's correlation. A p-value of < 0.05 was considered as significant.

6.2.3 Results

Demographics

Thirty-seven non-duplicate patients were identified during the period 1993 – 2008. Among them, 22 were males (male/female ratio = 1.5:1). Mean age was 51.7 years (range: 19 – 78 years). Mean age for females was 49.1 years, males was 53.4 years (p

> 0.05). The age distribution followed a bimodal distribution curve, which peaked at 41-50 years and 61-70 years (figure 6.1). The yearly trend of Cryptococcosis cases was shown in figure 6.2.

Clinical characteristics

The clinical characteristics are tabulated in table 6.1. Of the 37 infected patients, 27 (73%) had the organism recovered from cerebrospinal fluid. The remaining cases were recovered from blood cultures (six cases), pulmonary specimens (2 cases, bronchoalveolar lavage and lung biopsy), CAPD fluid (1 case) and vitreous fluid (1 case). Commonest presenting symptoms were fever (62.2%), headache (29.7%) and mental confusion (16.2%). The mean length of stay in hospital was 35 days (range: 1 – 137 days), the mean follow up time was 956 days (range: 1 – 3890 days).

Among the 27 meningoencephalitis patients, the mean CSF WBC count was 76/mm^3, (range: 5–309/mm^3). Lymphocytosis was observed in 19 of them (70.4%). India ink was positive in 17 (63%), negative in 8 (30%), and not performed in 2 cases. CSF:serum glucose ratio ranged from 0.07 to 0.61 (mean = 0.25). Mean CSF protein levels was 1.47g/dL (range 0.36 – 2.9g/dL). Blood cultures were simultaneously positive in 3 patients (1 SLE with ESRF, 1 SLE with carcinoma of breast, and 1 with carcinoma of breast). At diagnosis, CSF and serum cryptococcal antigen titres were

measured in 18 patients. Median CSF cryptococcal antigen titre was 1:256 (n=18, range: 1:4 – 1:4096). Median of serum cryptococcal antigen was 1:256 (n=18, range: 0 – 1:8192). Nine of them had low serum cryptococcal antigen titres (less than or equal to the median value), and the remaining nine patients had high serum antigen levels. The group with low serum antigen levels also had lower level of CSF antigen levels, they were more likely to survive ($p < 0.05$) at the end of analysis. Males were more common in the high serum antigen group ($p < 0.05$). Other characteristics did not differ significantly between the two groups (table 6.2). Serial (serum and/or CSF) cryptococcal antigen monitoring was performed in 6 survivors (figure 6.3). The rate of decline of CSF cryptococcal antigen titres had no significant correlation with that of serum antigen titres (Pearson's correlation $r = 0.49$ [95% confidence interval: -0.53 to 0.93], $p = 0.32$, figure 6.4). High baseline CSF or serum antigen titres (more than median of 1:256) did not associate with higher mortality rate at 2 weeks or 10 weeks ($p > 0.05$). On the other hand, high serum antigen titres achieved statistical significance when assessing mortality at the end of analysis period ($p < 0.05$). Raised intracranial pressure was observed in 11 patients, 6 (22.2%) suffered from hydrocephalus which required neurosurgical intervention with shunt insertion. Two meningitis patients were also co-infected with pulmonary tuberculosis, which was only confirmed by culture obtained from post-mortem. All meningitis patients received antifungal treatment with

amphotericin B and 5-FC (n=18, 66.7%), amphotericin B and fluconazole (n=1, 3.7%); or amphotericin B alone (n=8, 29.6%). Seventeen patients (45.9%) survived the infection episode.

Of the 10 non-meningeal cases, the one with vitreal infection and another with peritoneal infection (while on peritoneal-dialysis) were managed on fluconazole alone. They both survived. On the other hand, five out of six cryptococcaemic cases had the disease confirmed post-mortem and thus treatment was not initiated. The remaining one (HIV-positive) was treated with amphotericin B and 5-FC and survived. Of the two pulmonary cases, one was treated with amphotericin B followed by fluconazole, and the other with the addition of 5-FC. The overall survival rate for this group was 40% (n = 4)

HIV serology was performed on 23 patients out of the total 37 cases (62%), only 3 of them were seropositive and all 3 were males. One was aware of his HIV status prior to this hospital admission but refused medical treatment for 6 years. His CD4 count on admission was 6 cells/μL. The remaining two patients had their HIV infection diagnosed during these admissions. One had a CD4 count of 21 cells/μL. All of them had pulmonary co-infection with *Pneumocystis jirovecii* (*carinii*) or tuberculosis

(table 6.3). They all received antifungal treatment with amphotercin B with/without 5-FC, followed by fluconazole consolidation/maintenance therapy and HAART therapy. They all responded with clinical/microbiological/serological improvement. However, one of them presented with relapse of meningeal infection 6 months later (presenting with convulsion and positive growth of cryptococci from CSF) while on maintenance fluconazole and HAART therapy.

Of the remaining 20 HIV seronegative patients, 10 had identifiable underlying medical illnesses: diabetes mellitus (n = 3, 1 coexisted with polymyositis), one systemic lupus erythematosus (SLE), one metastatic carcinoma of breast, one diffuse large B-cell lymphoma, one pauci-immune necrotizing glomerulonephritis, one idiopathic thrombocytopenic purpura, one Hashimoto's thyroiditis and one hypertension (table 6.4). For the 14 patients whose HIV status was unknown, other risk factors present were SLE (n=3, 1 co-existed with carcinoma of breast, another with end-stage-renal-failure), malignancies (n=4), and cirrhosis (n=1).

6.2.4 Discussion

Although the number is too small to generalize a trend, the peak at 61 – 70 years represented the group of patients entering the extremes of age who are known to be at risk of cryptococcal infection. The other peak at 41-50 years probably coexists with the median of onset of NPC, SLE and malignancies (Kiertiburanakul 2006). As reported by Pappas *et al*, slight male age predominance was observed in our series (Pappas 2001). Majority of cases presented with fever, headache and confusion, but atypical presentation such as blur vision, cranial nerve palsy, cavitatory lung lesion, were also present in our series.

India ink is the classical way of diagnosing cryptococcal meningoencephalitis. Jongwutiwes *et al* reported a lower positive rate in HIV-negative patients (50%) when compared to HIV-infected patients (80%) (Jongwuitwes 2008). Kiertiburanakul *et al* also reported that India ink staining was positive in only 55% of cases (Kiertiburanakul 2006). Our results were consistent with their findings, highlighting that a negative result could not readily ruled out the diagnosis in a population in which HIV prevalence is low. In such situation, cryptococcal antigen testing would be a reasonable adjunct in confirming the diagnosis. Repeated lumbar punctures to collect more CSF may also be warranted. Six of our patients presented with low CSF WBC (< 10/mm^3), one was as

low as 5/mm^3. Such CSF WBC findings would generally be considered as normal (Seehusen 2003), resulting in a delay in diagnosis. The mean peripheral lymphocyte count of them was not significantly different from patients with raised CSF WBC, thus cannot explained the low inflammatory response in the brain. The CNS system is immunoprivilieged, activation of inflammatory cascade requires the presence of antigen presenting cells and cytokines. However, certain interleukins were known to suppress such response, including IL-10. It would be of interest to explore these inflammatory responses in meningitis patients were low CSF WBC.

The rate of decline of cryptococcal antigen titres showed no correlation between CSF and serum, suggesting that the clearance of cryptococcal polysaccharide was variable in individual patients and body compartments. Moreover, baseline CSF antigen levels did not predict outcome at 2 or 10 weeks in these patients. Therefore, although CSF antigen titres had been suggested to play a role as prognostic marker, our results did not support this notion. High baseline serum antigens titres, on the other hand, appeared to associate with final mortality at the end of the analytic period, probably reflecting the inability of the immune system to limit the disease inside the nervous system persistently. In another study of 68 patients with cryptococcal meningitis, monitoring of antigen levels was unhelpful in predicting the response of the treatment (Brouwer 2005). While the antigen levels did decline as observed in the

survivors, the precise role of cryptococcal antigen monitoring needs to be further clarified.

Neurological complication such as hydrocephalus was found in 40.7% of our patients, surgical intervention was required in half of them. Two patients actually presented with hydrocephalus to the neurosurgeons directly for surgical intervention. Thus, it is essential to rule out cryptococcal infection in any patient with unexplained raised intracranial pressure. The management of raised intracranial pressures can be achieved by repeated lumbar punctures, or by putting in a ventriculo-peritoneal CSF shunt. Even with initial normal intracranial pressures, hydrocephalus can still develop during the course of therapy.

Cryptococcus neoformans can be further divided into several variants such as *Cryptococcus neoformans var neoformans* (serotype A, D) and *Cryptococcus neoformans var gattii* (serotype B, C). The distinction can be accomplished by serotyping or dot blot immunoassay. *Cryptococcus neoformans var. gattii* had been reported to infect mostly immunocompetent patient, and could be a potential explanation for our patients with no risk factors. Unfortunately we had not adopted these methods and were unable to distinguish them routinely. However, based on the geographical distribution, it is probable that our cases were due to serotype A, which was the predominant type in Asian countries. It would be useful to adopt other simple

measures such as canavanine glycine bromothymol blue (CGB) agar as a routine service in the laboratory. Alternatively, strains should be archived for subsequent analysis in a batch-wise manner.

In our setting, the readily available amphotericin B formulation is still the deoxycholate form. Lipid formulations require special purchase by the patient, and thus are not commonly prescribed. As a result, we were unable to address whether lipid-based amphotericin B has better efficacy and clinical outcome (Baddour 2005; Saag 2000). The addition of 5-FC is an independent factor for favorable outcome. Effort should be made to ensure that this is given to ensure speedy sterilization of CSF.

While awareness concerning HIV/AIDS has improved, there is evidence that the public is losing interest in this issue, and safe sexual practices are not always followed (Lau 2002a, 2002b, 2002c). In locality where HIV prevalence is perceived to be low, the diagnosis of HIV may not be suspected initially (Wong 2001). In the 3 patients who were HIV seropositive, 2 were newly diagnosed during these episodes, another one had defaulted treatment with a CD4 count of only 6 cells/uL. In the latter patient, the acute infection was successfully managed with amphotericin B and 5-FC, with microbiological and serological response (CSF antigen titres declined from 1: 1024 to1:256). He relapsed while on maintenance fluconazole of 200mg per day and HAART treatment, together with microbiological (positive india ink and culture) and

serological proof (CSF titre raised to 1:1024). It was possible that the relapse is due to immune reconstitution syndrome, however, it was difficult to ascertain as fluconazole MIC was not available, and antifungal resistance could not be completely ruled out. Patient compliance with the fluconazole therapy may also be a concern. A larger cohort would be needed to systematically assess these events in HIV positive patients.

There were several limitations in our study. The sample size was relatively small, reflecting that the overall prevalence of *Cryptococcus* infections was still an uncommon entity. The increase in cryptococcal infections observed in other countries was mostly contributed by the population of patients with AIDS. Geographical locations with low HIV infection rate did not observed such a surge. Moreover, our series of patients were diagnosed by microbiological methods; some cases will be missed as they may be diagnosed by histological proof only.

In summary, cryptococcal infection is still an uncommon infection in low-HIV prevalence area. Meningoencaphalitis and the need of neurosurgical intervention were common. Baseline CSF cryptococcal antigen testing has a role in diagnosis; however, its use in monitoring of disease response appeared to be limited.

Figure 6.1. Age distribution of cryptococcal infections.

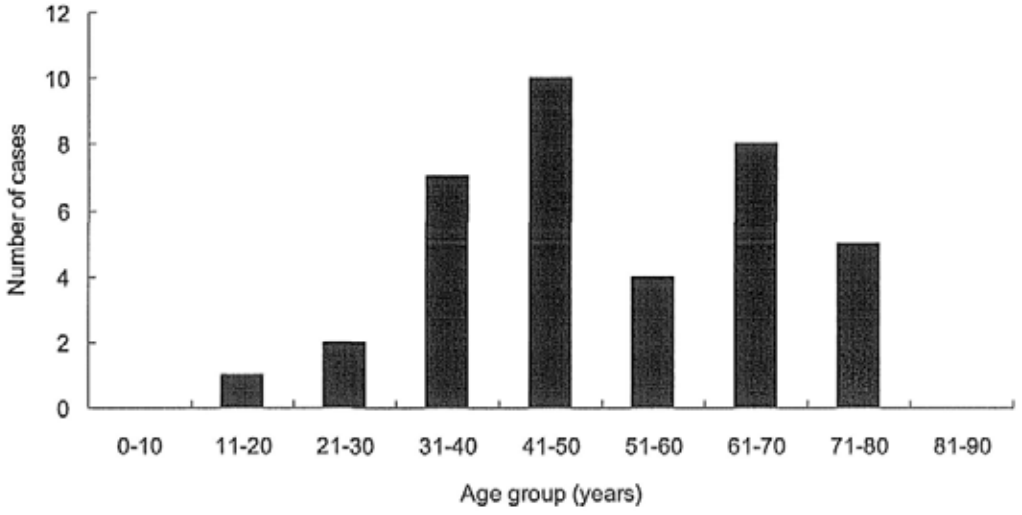


Figure 6.2. Yearly trend of cryptococcal infections from 1993 to 2008.

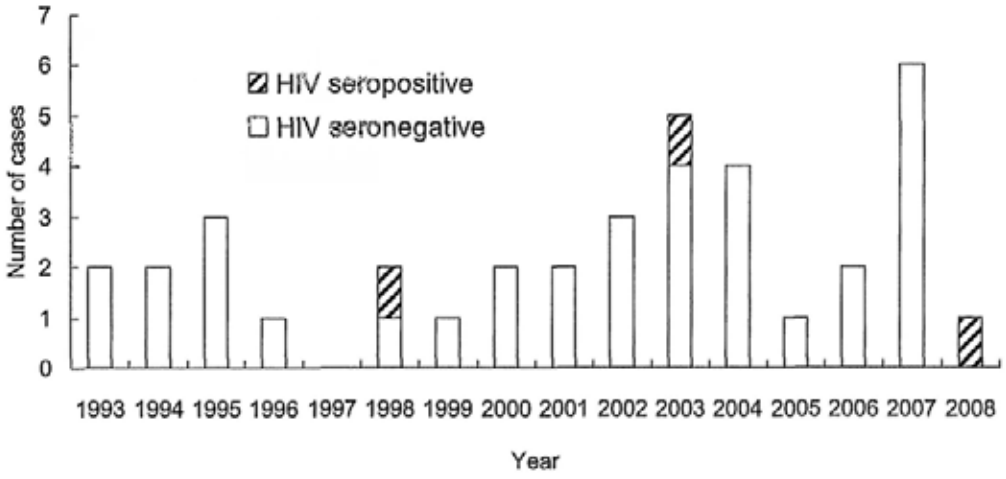


Figure 6.3. Time trend of serum and CSF cryptococcal antigen titres in surviving cryptococcal meningoencephalitic cases.

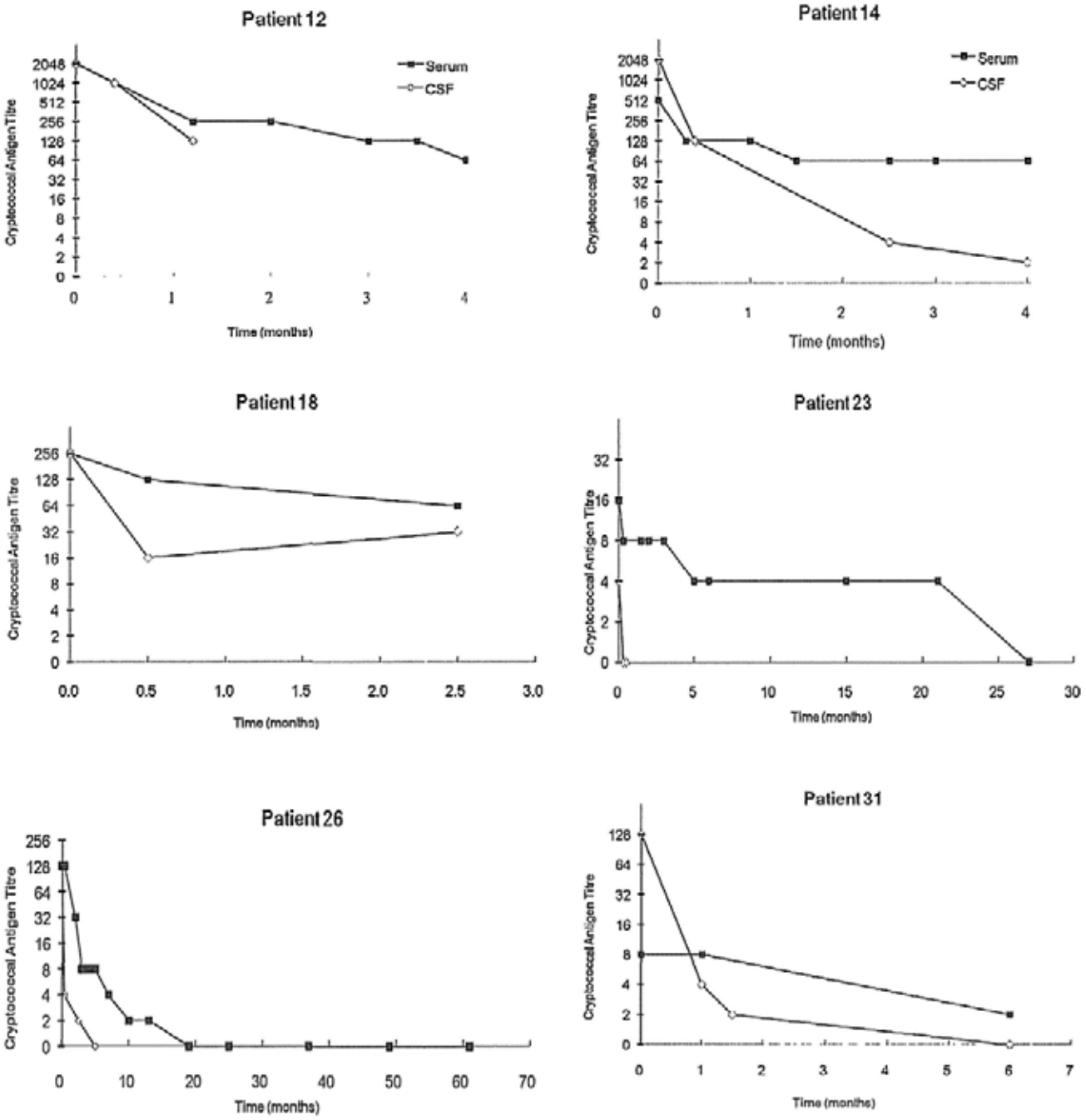


Figure 6.4. Lack of correlation between rate of decline of CSF and serum cryptococcal antigen titres in surviving meningoencephalitic patients.

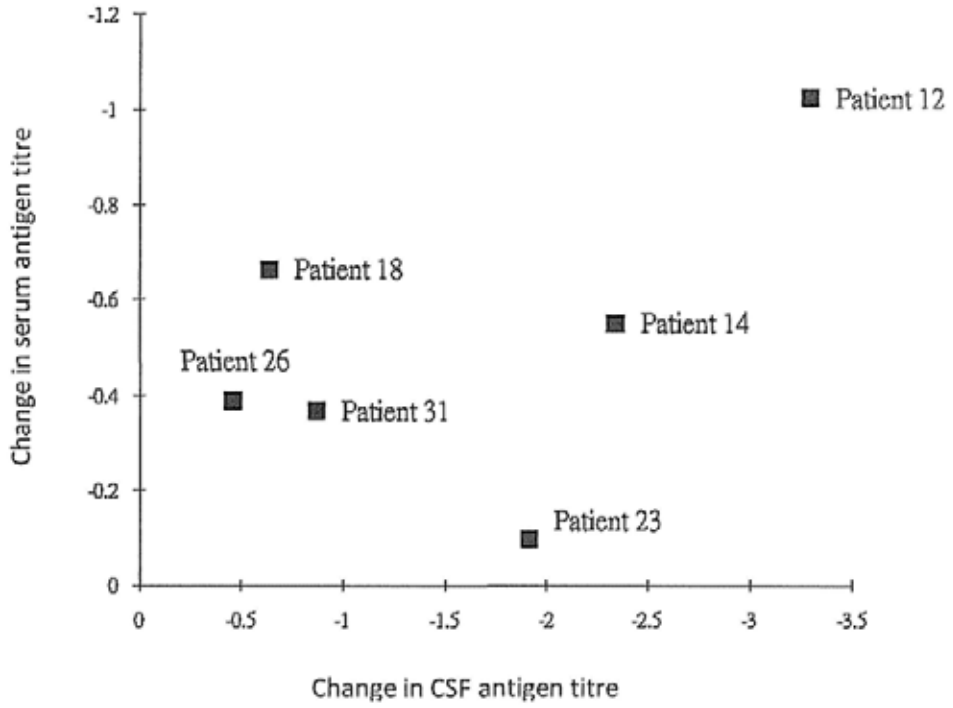


Table 6.1. Demographics and clinical features of patients with Cryptococcal infections ($n = 37$).

Demographics	
Age (years)	
Mean (range)	51.7 (19-78)
Male : Female	1.5 : 1
Site of infections	
Central nervous system	27 (73%)
Blood stream infections	6 (16.2%)
Pulmonary	2 (5.4%)
Others *	2 (5.4%)
Clinical features	
Fever	23 (62.2%)
Neurological	
Headache	11 (29.7%)
Confusion	6 (16.2%)
Visual disturbance	3 (8.1%)
Vomiting	1 (2.7%)
Psychiatric disturbance	1 (2.7%)
Cranial VI nerve palsy	1 (2.7%)
Pulmonary	
Pneumonia	3 (8.1%)
Cavitatory lesion	1 (2.7%)
Meningoencephalitis cases *(n = 27)*	
Laboratory Investigations	
Peripheral total WBC x 10^9/L [mean (range)]	9.2 (1.9 – 18.9)
Peripheral lymphocyte count x 10^9/L [mean (range)]	1.09 (0.3-2.8)
Cryptococcal antigen titre (at diagnosis)	
CSF [median (range)]	256 (0 – 2048)
Serum [median (range)]	256 (4-8192)
CSF WBC/mm^3 [mean (range)]	76 (5 – 309)
Complications	
Raised intracranial pressure	11
Neurosurgcial intervention (shunt) required	6

* 1 CAPD and 1 vitreous case.

Table 6.2. Characteristics of cryptococcal meningoencephalitis with low and high serum antigen levels.

	Low serum cryptococcal antigen titre (≤1:256, n=9)	**High serum cryptococcal antigen titre (>1:256, n=9)**	***p*-value**
Age (years) [mean (range)]	52.6 (25 – 64)	46.4 (29-75)	>0.05
Male (*n*)	3	7	<0.05
Presence of predisposing factors (*n*)	5	6	>0.05
CSF antigen titre [median (range)]	32 (0-128)	1024 (512-4096)	<0.05
CSF WBC [mean (range)]	103 (7-139)	53 (12-104)	>0.05
Peripheral WBC x 10^9/L	9.2 (6.6-12.2)	10.2 (1.9-16.7)	>0.05
Peripheral lymphocyte x10^9/L	1.2 (1.0-1.4)	1.14 (0.3-2.0)	>0.05
Complication (*n*)			
Raised intracranial pressure	2	6	>0.05
Neurosurgcial intervention (shunt) required	2	3	>0.05
Survival at end of analysis (*n*)	8	4	<0.05

Table 6.3. Characteristics of HIV-infected patients with cryptococcal infection.

Case	Sex	Underlying illness	Diagnosis on admission	CD4 count (cells/μL)	Site	Co-infection	Outcome
10	M	Nil	Pneumonia	Not available	Blood culture	*Pneumocystis jirovecii*	Survive
17	M	HIV (defaulted treatment)	Confusion	6	Cerebrospinal fluid	*Pneumocystis jirovecii*	Relapse 6 months later
24	M	Nil	Pneumonia	21	Bronchoalveolar lavage	Tuberculosis	Survive

Table 6.4. Characteristics of HIV-negative patients with cryptococcal infection with underlying medical illnesses.

Case	Sex	Underlying illness	Medication	Site	Outcome
13	M	Pauci-immune necrotizing glomerulonephritis	Azathioprine, prednisolone	CSF	Survive
18	F	Systemic lupus erythematosus	Prednisolone, cyclophosphamide	CSF	Survive
19	F	Diffuse large B-cell lymphoma	etoposide, ifosfamide, methotrexate	Vitreous	Survive
29	F	Idiopathic thrombocytopenic purpura	None	CSF	Survive
30	F	Diabetes mellitus	Isophane insulin	CSF	Survive
31	M	Diabetes mellitus	Gliclazide	CSF	Survive
32	F	Metastatic carcinoma of breast	None	CSF	Death
33	F	Hashimoto's thyroiditis	Thyroxine	CSF	Survive
35	M	Polymyositis, Diabetes mellitus	Prednisolone, azaththioprine, isophane insulin	CSF	Death
36	M	Hypertension	Nifedipine	CSF	Survive

6.3 Study Two: *Pneumocystis jirovecii (carinii)* pneumonia in a tertiary referral hospital.

6.3.1 Abstract

A retrospective review of patients diagnosed with *Pneumocystis jirovecii* (formerly *Pneumocystis carinii*) pneumonia (PCP) from 1993 to 2008 at the Prince of Wales Hospital in Hong Kong is presented. Thirty patients were identified. Sixteen (53.3%) were co-infected with Human Immunodeficiency Virus (HIV). All the remaining 14 non-HIV infected patients had underlying diseases: 4 post-renal transplant recipients, 3 haematological malignancies, 3 auto-immune diseases, 2 renal diseases, 1 hepatocellular carcinoma, and 1 congenital cytomegalovirus (CMV) disease. CMV co-infection was observed in 5 patients. All patients received co-trimoxazole therapy, with intolerance observed in 5. This included 1 with glucose-6-phosphate dehydrogenase (G6PD) deficiency, 2 with repeated vomiting, 1 with skin rash and 1 with renal impairment. Overall crude mortality was 26.7%. The results suggest that apart from being a common infection for patients with HIV infection, PCP could occur during the course of many immunosuppressive diseases and therapies. The mortality of PCP is high despite appropriate treatment. The role of prophylaxis in at risk (non-HIV) populations should be assessed.

6.3.2 Methods

Patients and case definitions

A retrospective review was conducted to analyze the characteristics of the patients suffering from PCP from 1993 to 2008 through a retrospective case review. Cases were identified from the laboratory log of the Microbiology Department of the Chinese University of Hong Kong. The laboratory provides diagnostic and consultative services to the Prince of Wales Hospital (a university affiliated tertiary referral hospital) and the Northern District Hospital (since 2002). Laboratory results were archived electronically by in-house software. *Pneumocystis jirovecii* infection was defined by positive staining results ($\geq$ 1 positive cyst) from toluidine Blue O staining of bronchoalveolar lavage specimens or lung biopsy specimens together with compatible clinical features. Non-sterile respiratory specimens such as sputum and tracheal aspirate would be not accepted for staining. Pulmonary involvement was considered clinically compatible when fever of $\geq$ 38.5^{o}C and radiological changes were present. Clinical outcome was assessed on the date of death, last follow up or on Dec 31, 2008, whichever is later. We have focused on the epidemiology, underlying diseases, treatment and mortality of these PCP patients. Results were entered into a standard log.

6.3.3 Results

Thirty patients with PCP were identified from 1993 to 2008, of which 23 were males (M:F = 3.4:1). Mean age of the patients was 44.4 years (range: 4 months – 67 years). Mean age of female patients was 44.8 years, and that of male patients was 43.8 years. The yearly trend was shown in figure 6.5. From 2000 to 2008, the total number of request for PCP investigations was 793, a gradually increasing trend was observed (figure 6.6).

Sixteen (53.3%) patients were HIV positive, only one of them was female. Three patients were aware of their HIV seropositive status prior to the admission with PCP. Of the 12 patients who had CD4 cell count measured, the mean was 36 cells/μL (range: 3 – 108 cells/μL). All remaining patients had their HIV status diagnosed during these admissions with PCP.

All HIV seronegative patients (n = 14) had underlying diseases. Four were renal transplant recipients, 3 with haematological malignancies (2 non-Hodgkin's lymphoma (NHL), 1 T-cell lymphoma), 3 with autoimmune diseases (2 systemic lupus erythematosus, 1 dermatomyositis), 2 with renal diseases (1 nephrotic syndrome,

1 glomerulonephritis), 1 with advanced hepatocellular carcinoma, 1 was an infant with congenital CMV disease, together with necrotizing enterocolitis.

All HIV seronegative patients with PCP (except the infant) were receiving immunosuppressive therapies at the time of diagnosis. The drugs involved included prednisolone (daily dose range: 15mg – 100mg), cyclophosphamide, cyclosporine A, azathioprine, doxorubicin, vincristine, chlorambucil, cytarabine, mercaptopurine, methotrexate, mycophenolate mofetil and rituximab (Table 6.5).

Eleven of the 14 HIV seronegative patients acquired PCP within 7 months after the diagnosis of the underlying diseases. In particular, all four post renal transplant recipients had commenced immunosuppressive therapy within 6 months prior to the diagnosis of PCP.

All PCP patients were started with cotrimoxazole. The commonest dose used was 3840 mg per day (n = 8) in divided doses. For adult patients, the highest daily dose of cotrimoxazole used was 9600 mg; while the lowest dose was 960 mg. The single infant was given 360 mg. Among the adult patients, 19 were given steroid adjuvant therapy, including prednisolone in 17 patients and hydrocortisone in 2 patients. One

patient was switched to pentamidine after 4-days of cotrimoxazole (3840 mg per day) treatment due to slow clinical improvement. Another patient was identified to have G6PD deficiency. He was initially treated with cotrimoxazole, but was subsequently changed to trimethoprime after just one dose. No drug induced haemolysis was detected in this patient. Two patients suffered from repeated vomiting while on treatment (1 on cotrimoxazole, 1 on trimethoprime), but managed to continue with their therapy. A further patient developed skin rash while on cotrimoxazole and was switched to pentamidine. Another patient initially treated with cotrimoxazole had to decrease the dosage from 3600 mg per day to 2400 mg per day due to renal impairment. Severe side effects such as Stevens-Johnson syndrome were not observed in any patient.

No patient was given primary prophylaxis. All surviving HIV seropositive patients received cotrimoxazole or pentamidine as secondary prophylaxis. For the others, only 4 patients (2 renal transplant recipients, 1 SLE, 1 NHL) were given secondary prophylaxis with cotrimoxazole.

Six patients were observed to have concomitant bacterial pneumonia (1 with *Pseudomonas aeruginosa*, one with *Streptococcus pneumoniae*, 2 with

Mycobacterium tuberculosis, 2 with *Mycobacterium avium/intracellulare*). Five patients had concomitant CMV pneumonitis. Fourteen patients required admission to the intensive care unit and 7 required assisted ventilation. Eight patients died and six were HIV seronegative. Overall crude mortality was 26.7%. Patients with concomitant bacterial or viral pneumonia had a mortality of 54.5%. For those requiring intensive care, the mortality was 50%. For the group of patients treated with steroid adjuvant therapy, the mortality was 21.1%; mortality of those without steroid therapy was 36.4%. The mean age of the patients who died was 44.7 years, and that of the survivors was 44.0 years. No recurrence of PCP was observed during the follow up period (mean duration = 6.5 years; range: 4 months – 14 years).

6.3.4 Discussion

Pneumocystis jirovecii is a fungus capable of causing life-threatening pneumonia in patients with impaired cellular immunity (Cailliez 1996; Cunha 2001). It is one of the common opportunistic infections found in HIV infected patients. Primary and secondary prophylaxis has been advocated to prevent the infections in AIDS patients (Barry 2001; Phair 1990; Schliep 1999). On the contrary, much less is known with PCP in non-HIV infected patients. Hong Kong has a very low prevalence of HIV

infection, with an estimated prevalence of less than 0.1% (WHO 2000). Therefore, the spectrum of PCP infection may be different from populations in which HIV infection rates are higher.

In our study, 16 (53.3%) of the 30 PCP patients were HIV infected. Other researchers had reported variable rates of HIV co-infections. A 10 years case-review study done in Sydney found that 69.6% PCP patients had HIV co-infection (Gerrard 1995), whereas another done in Sweden showed that only 25% were HIV co-infected (Mikaelsson 2006). This difference is probably due to difference in HIV prevalence, as well as presence of other risk factors.

Patients' age distribution followed a normal distribution curve, with a peak at the age range of 40-49 years. This probably reflects the general epidemiology of the underlying diseases in our patients, for example, renal transplant recipients and SLE. However, the number of the patients is too small to draw a definitive statistical conclusion. Among all the cases, one was an infant with congenital CMV infection. This infant was regarded as otherwise immunocompetent and had been reported previously by Leung *et al* (Leung 2000). They proposed that the co-infection with CMV and trace element deficiency with zinc may have compromised the infant's

immune defense.

The underlying risk factors for PCP found in this study were similar to those reported in other studies (Arend 1995; Saksasithon 2003; Sepkowitz 2002; Roblot 2002;). All HIV seronegative adult patients had chronic underlying diseases that required immunosuppressive therapies. Studies have shown that chronic steroid use predisposes patients to a variety of infections by impairing their host defense mechanisms. Stuck *et al* suggested that the risk of infections was greater when a daily dose of >10 mg prednisolone was given (Stuck 1989). Since steroid can be used to treat a wide spectrum of diseases (including organ rejection therapy and autoimmune diseases), one should be aware of the increased risk of infection when larger doses are used (Klein 2001).

Despite the underlying risk factors, none of the patients was receiving primary prophylaxis. This is particularly worrying as three patients were known to be HIV seropositive prior to presentation with PCP, and none of them received HAART nor prophylactic therapy for opportunistic infections (Klein 1992; Palella 1998; Vilar 1999). The remaining HIV infected patients were only diagnosed at the presentation with PCP. This reflects another worrying aspect: in a locality with a low HIV

prevalence, patients are relatively unaware of the risk, resulting in late presentation (Wong 2003). Chemoprophylaxis has also been shown to be effective in patients with other risk factors, such as cardiac and renal transplantation (Gordon 1999; Higgins 1989; Olsen 1993). Since the mortality of PCP remains high even with appropriate treatment, prevention may be the best way to reduce the number of deaths due to this disease. Prophylaxis against *Pneumocystis* had been advocated in haematopoietic stem cell transplant recipients and those who received prednisoslone of > 20 mg for over 1 month (CDC 2000, Sepkowitz 2002). Green *et al* also advocated the used of prophylaxis if the risk of Pneumocystis infections is higher than 3.5% (Green 2007). Large controlled trials would be warranted to assess the usefulness of primary prophylaxis in other non-HIV immunosuppressed patients.

There were no severe side effects associated with the use of cotrimoxazole in our group of patients. Only 3 of 30 patients failed to continue initial therapy (due to intolerance). G6PD deficiency is a relatively common condition in Hong Kong, with an incidence of 4.5% in males, and 0.36% in females (Li 1990). In our study group, only one patient was found to have G6PD deficiency. This suggests that cotrimoxazole is a generally well tolerated medication in our population.

Overall crude mortality in our study was similar to that reported in other studies (28-38%) (Arend 1995; Fernandez 1995; Gerrard 1995; Roblot 2002; Saksasithon 2003). The mortality was higher in patients with concomitant viral pneumonitis or bacterial pneumonia. This suggests that respiratory secretions and other appropriate specimens should be sent for the detection of other bacterial or viral pathogens. This allows early institution of antibiotic or antiviral to treat concomitant infection.

Previous studies suggest that corticosteroids should be used in moderate to severe PCP as adjuvant therapy because it can improve survival and prevent the development of acute respiratory failure (Montaner 1990). In our study, mortality among patients with steroid adjuvant therapy was slightly lower (21.1%) than the overall mortality (26.7%), but it did not achieve statistical significance. This may be due to the small sample size of our study group, therefore failing to demonstrate the beneficial effect of adjuvant steroids. Alternatively it may reflect the increased level of severity in those given steroid adjunct therapy. A larger sample size would help to better delineate the nature of the differences in mortalities.

There were limitations to this case series. As cases were confirmed by toluidine blue O stain, some cases may be missed and only be diagnosed on clinical grounds or

by histological proof. Moreover, the number of cases was still small to confirm any particular trend.

These results suggested that apart from being a common infection in patients with AIDS, PCP could also occur during the course of many immunosuppressive diseases and therapies. The mortality of PCP was high even with appropriate treatment. The benefit of prophylaxis (in non-HIV patients) should be further assessed. Even in a locality with low HIV prevalence, improved education to raise the awareness of the population with earlier detection of HIV infection could also be beneficial.

Table 6.5. Underlying diseases and immunosuppressive agents used in all HIV seronegative *Pneumocystis jirovecii* pneumonia patients.

Case	Underlying Disease	Immunosuppressive agents used
1, 2	Non-Hodgkin's lymphoma	Pred, CTX, DOX, VCR
3, 4, 5, 13	Renal Transplant recipient	Pred, CyA, AZA, MMF
6	T cell lymphoma	Pred, CTX, Ara-C, 6-MP, MTX
7	Dermatomyositis	Pred
8, 14	Systemic Lupus Erythematosus	Pred, CTX, Rit
9	Hepatocellular carcinoma	Pred, DOX
10	Glomerulonephritis	Pred, CTX
11	Nephrotic syndrome	Pred, CLB
12	Congenital CMV infection	none

Abbreviations: Pred, prednisolone; **CTX**, cyclophosphamide; **CyA**, cyclosporin A; **AZA**, azathioprine; **DOX**, doxorubicin; **VCR**, vincristine; **CLB**, chlorambucil; **Ara-C**, cytarabine; **6-MP**, mercaptopurine; **MTX**, methotrexate; **MMF**, mycophenolate mofetil; **Rit**, Rituximab.

Figure 6.5. Yearly trend of number of *Pneumocystis jirovecii* pneumonia cases.

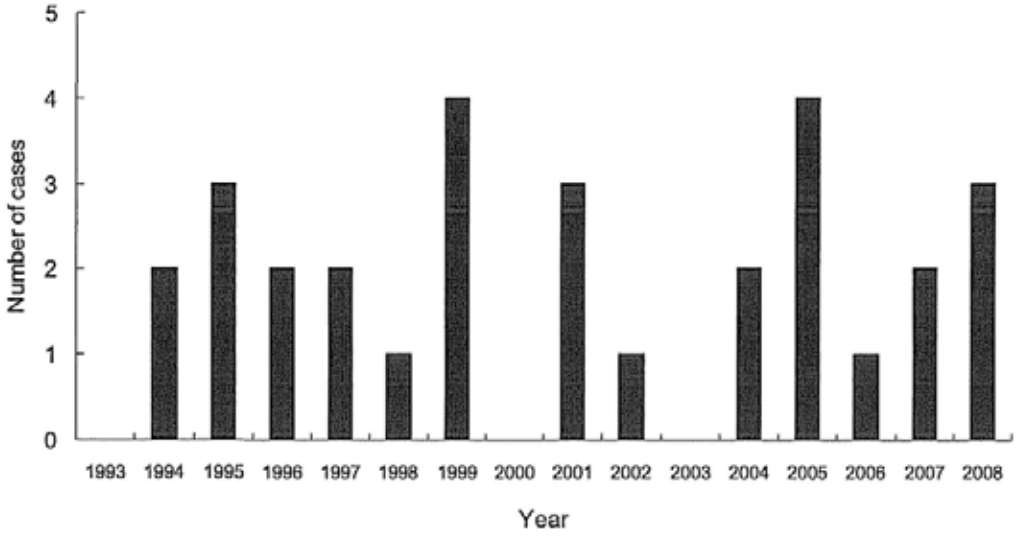
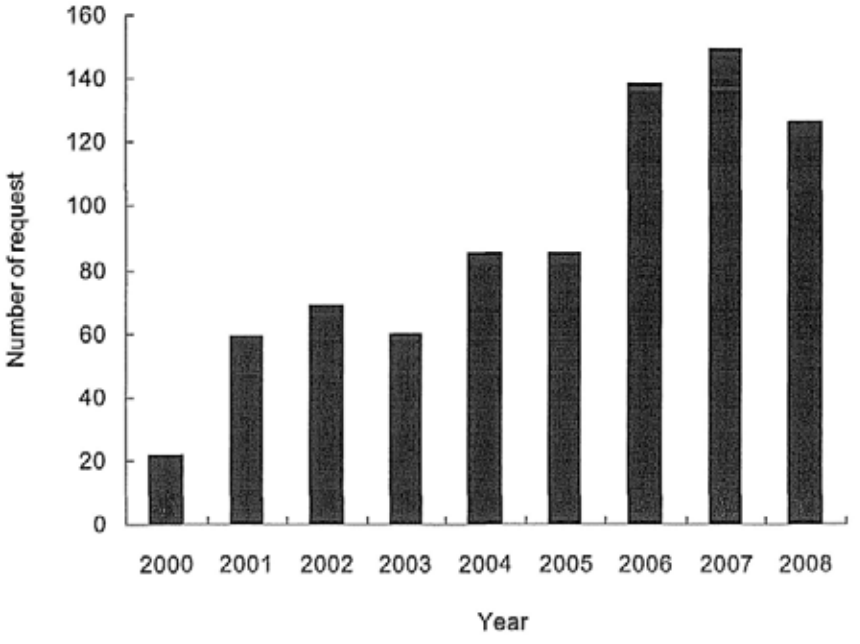


Figure 6.6. Yearly trend in the number of request for *Pneumocystis jirovecii* pneumonia investigations from 2000 to 2008.



6.4 Study Three: Invasive *Candida* blood stream infections from 1993 to 2008 – a retrospective review.

6.4.1 Abstract

Candida albicans and *Candida* non-albicans are commonly found as commensals in human body. Hence, their significance as opportunistic pathogens is often neglected. A retrospective review was undertaken to analyse the clinical characteristics of invasive blood stream infections caused by *Candida* species. The review was conducted on patients suffering from invasive *Candida* blood stream infections at the Prince of Wales Hospital in Hong Kong from 1993 to 2008. Cases were identified from the laboratory log of the Microbiology Department. During this period, the laboratory utilized an automated blood culture system for blood culturing. A total of 480 candidaemic episodes involving 478 patients were identified. Among these, 259 (52.2%) were *C. albicans*. For the remaining *Candida* non-albicans species, 60 (12.1%) were *C. parapsilosis*, 105 (21.2%) were *C. tropicalis,* 50 (10.1%) were *C. glabrata.* This is consistent with the global trend whereby *C. albicans* is no longer the predominant organism found in invasive disease. Other *Candida* species, with variable amphotericin B and azoles susceptibilities, had emerged. This has an impact on the most appropriate choice of antifungal therapy.

6.4.2 Methods

A retrospective review was conducted to analyze the characteristics of the patients suffering from candidaemia from 1993 to 2008 through a retrospective case review. Cases were identified from the laboratory log of the Microbiology Department of the Chinese University of Hong Kong. The laboratory provides diagnostic and consultative services to the Prince of Wales Hospital (a university affiliated tertiary referral hospital) and the Northern District Hospital (since 2002). Laboratory results were archived electronically by in-house software. Positive cases were defined as growth of *Candida* organisms from blood cultures. Recovery of the same *Candida* species within 30-day period was considered as one episode. Relapse was defined as recurrence of the same candidal organism from blood culture after completion of anitifungal treatment with satisfactory clinical response. The laboratory employed an automated blood culture system with Bactec 660 blood culture system (Becton Dickinson, Sparks, MD) from 1993 to 1997. From 1998 onwards, BacT/Alert system (Organon Teknika Corp., Durham, N.C.) was used. All routine blood cultures were incubated for at least 5 nights before being discarded as negative. For blood cultures specifically requested for fungal isolation, prolonged incubation for 21 days is performed. Organisms are identified by gram's stain

morphology, germ tube formation, carbohydrate assimilation test (API 20C AUX or 32C AUX, bioMerieux, Hazelwood, Mo.; Vitek 2, bioMérieux, Marcy l'Etoile, France), morphological appearance on Cornmeal agar and CHROMagar. The patient's case files were reviewed and results entered into a standard log and analysed.

6.4.3 Results

In the period 1993 - 2008, a total of 478 patients and 480 episodes were identified.

Among these, 259 (52.2%) were *C. albicans*, followed by *C. tropicalis* (105 episodes, 21.2%), *C. parasilopsis* (60 episodes, 12.1%) and *C. glabrata* (50 episodes, 10.1%). The remaining species constituted less than 5% of all candidaemic episodes. The male to female ratio was 1.6:1. Forty-five patients were neonates. One hundred ninety-two patients were diagnosed in ICU. Peak age occurred at < 1 yr of age and at 70 - 79 yr. Mortality increased with age, in those >80 years old, the mortality was 71%. (figure 6.7). Throughout the years covered, there was a gradual increase in the number of candidaemic episodes (figure 6.8). *C. albicans* constituted half of the cases among various age groups, with the exception of the age 10-19,

where *C. tropicalis* predominated (figure 6.9). *C. glabrata* showed a gradual increase towards the elderly age group (up to 20% for those > 80 years of age). Vast majority of the patients had risk factors such as presence of central venous catheters (68%) and prior use of multiple antibiotics (52.7%). Other risk factors such as malignancies (27.4%), recent surgery (27.8%), total parenteral nutrition (19.2%), diabetes mellitus (16.1%), use of chemotherapeutic agents (15.3%) and corticosteroids (7.5%) were also identified (table 6.6). Complications were uncommon. Only 1 patient developed endophthalmitis, another 4 patients with radiological evidence of hepatosplenic candidiasis.

Analysis of all bloodstream infections with respect to the cultured organisms was done for the year 2008 (figure 6.10). A total of 2585 positive blood cultures were obtained. *E. coli* is the commonest organism identified (34.2%), followed by *Klebsiella* species (14.2%), *Staphylococcus aureus* (methicillin-sensitive and methicillin-resistant) (12.4%), *Candida* species ranked at number 4 (5.2 %). No uniform usage of antifungal drugs was identified, regimens used include amphotericin B, fluconzole, voriconazole and caspofungin.

6.4.4 Discussion

Consistent with reports from other centres, *Candida* non-albicans has evolved to account for nearly half of all the candidaemic cases (Arendrup 2010; Lass-Flörl 2009). *C. tropicalis* was not common in patients less than 1 year old, but its prevalence increased sharply to 29% in the age group 1-9, and peaked at 47% (outnumbered *C. albicans*) in the group aged 10-19. It gradually decreased but remained at more than 10% among other age groups. *C. parapsilosis*, on the other hand, was common in age groups less than 9 year of age, and gradually decreased with age. *C. glabrata* was generally uncommon, but there was a gradual trend of increase towards the elderly (20% in those more than 80 years old). *C. glabrata* is of particular importance, because this organism can exhibit reduced azole susceptibility, thus requiring a higher dose of azoles or the need of alternative agents. Therefore, in the elderly with candidaemia, it is not desirable to wait for the laboratory to identify the organism before escalating treatment regimen. Prompt treatment with high dose azole, or the use of alternative agents should be considered. Advocates have suggested de-escalating strategy which employs the use of broader spectrum antifungal agents (such as echinocandin), and then to step down to a narrower agent when the organism is speciated (Pappas 2009).

Mortality was high in candidaemic patients, it rose from 25% in those less than 1 year old, to more than 70% in those more than 80 years old. There could be many reasons for this. Candidaemia may not be suspected initially; azole resistant species (such as *C. glabrata*) was not being aware of; persistent nidus of infections (such as vascular catheter) was not removed; or the host was extremely debilitated and the candidaemia was admittedly an end-of-life event.

C. parapsilosis ranked 3rd and constituted 12.1% of cases. This organism is associated with vascular catheter infections (Krcmery 2002, Trofa 2008). Thus prompt removal of the vascular catheters should be considered in these cases. However, vascular catheters, especially those central venous ones, are generally considered as 'precious'. There is great reluctance among clinicians to remove these catheters due to various reasons, such as difficult venous access and bleeding tendency. While these concerns are understandable, there were evidences to suggest that removal of catheter improved clinical outcome (Almirante 2005, Weinberger 2005). Alternatively, successful antibiotic lock therapy with intraluminal amphotericin B had been reported. Further clinical trials are warranted to assess its efficacy (Angel-Moreno 2005).

In fact, as a measure of the quality of medical care, guidelines on the management of fungal infections should be developed, implemented and audited

(Aliyu 2006, Denning 2003b; Schelenz 2009). With the risk factors identified in this study, one should include vascular catheter care, prudent use of antibiotics, early recognition of at risk patient groups and potential complications into these guidelines.

There appeared to be a gradual increase of cases from 1993 to 2008. This may be due to more intense use of therapeutic agents that compromised patients' immunity, as well as more liberal use of multiple antibiotics, and more invasive procedures being used on our patients.

Candida species ranked 4th commonest pathogens causing bloodstream infections in 2008, after *E .coli*, *Klebsiella species* and *Staphylococcus aureus*. This was consistent with the USA findings (Pfaller 1995b). Awareness should be kept in mind that candidaemia is no longer a rare disease.

There were limitations in this study, as it was a retrospective analysis, patients who had been treated empirically may not yield a positive blood culture. Those with deep-seated disseminated diseases may also be blood culture negative. Conventional blood cultures were also known to be not highly sensitive in confirming fungaemia.

Candidaemia is not an uncommon blood stream infection. *Candida* non-albicans has emerged and can outnumber *C. albicans* in young age groups. Early recognition of patients at risk, prompt institution of antifungal therapy and removal of vascular catheters are all crucial in the management if these patients.

Clinical guideline should be developed to improve the quality of health care.

Figure 6.7. Age trend of candidaemia and mortality rate at 30 days after onset of candidaemia.

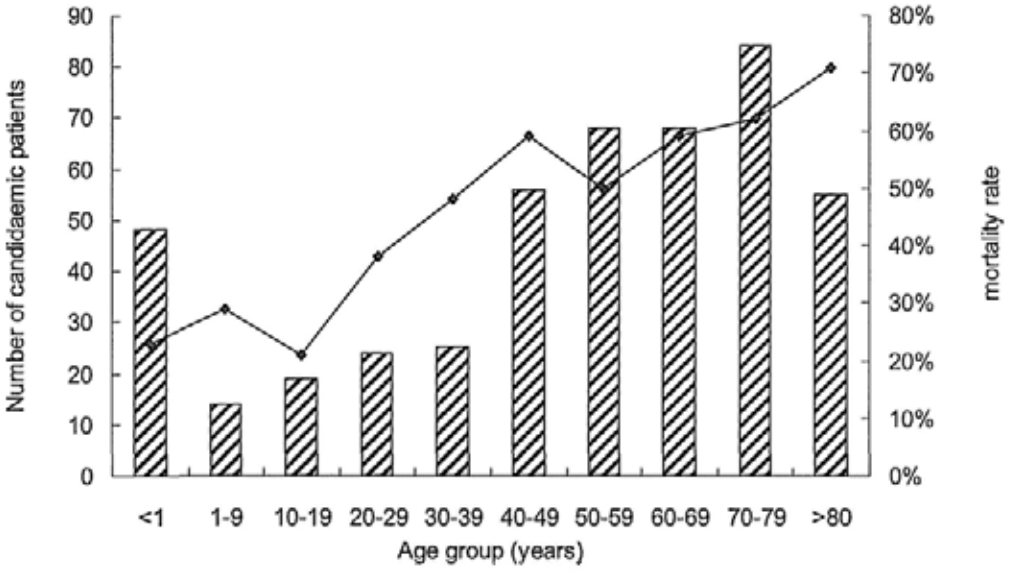


Figure 6.8. Yearly trend of candidaemia per 1000 admission.

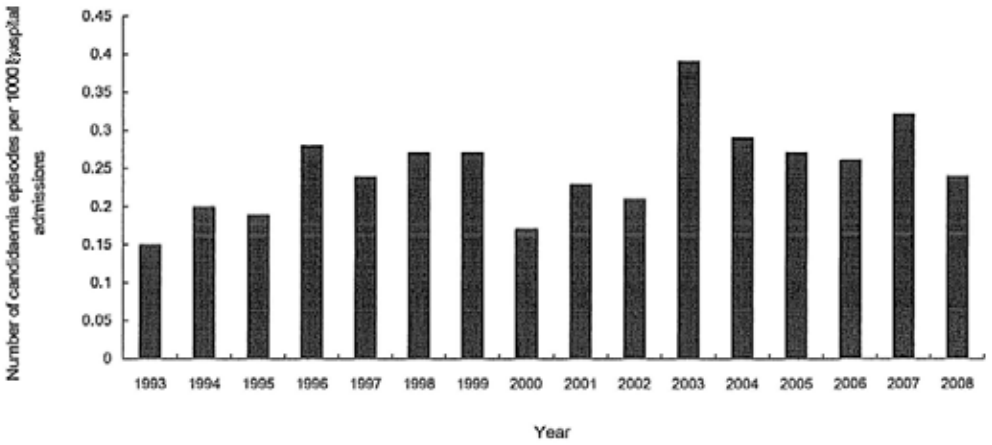


Figure 6.9. Age distribution of 4 Candida species.

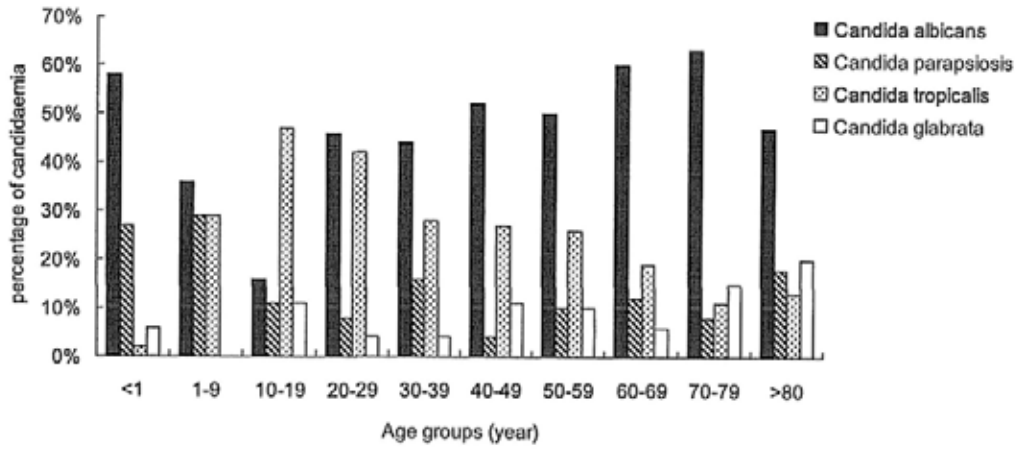


Figure 6.10. Ranking of blood culture isolates in 2008.

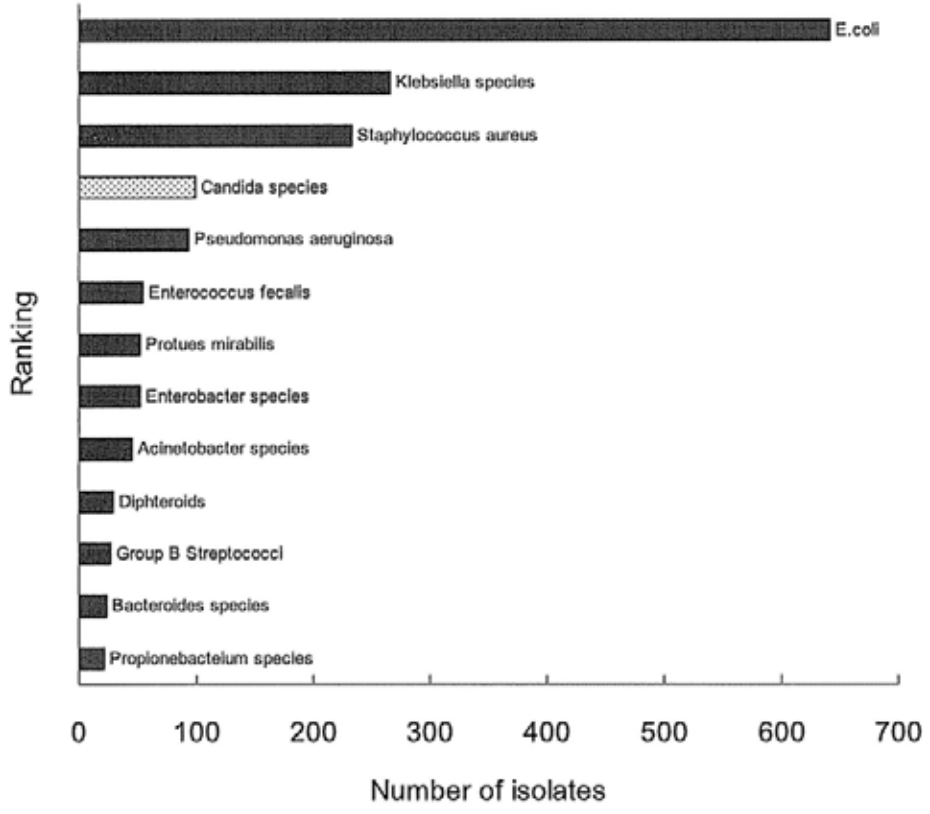


Table 6.6. Demographics, risk factors and clinical features in candidaemic patients.

Demographics (*n=478*)	
Age (years)	
Mean ± S.D.	50.5 ± 26.9
Male, *n* (%)	298 (62.3)
Risk factors, *n* (%)	
Diabetes mellitus	77 (16.1)
Malignancies	131 (27.4)
Transplant recipients	17 (3.6)
Burns	12 (2.5)
Corticosteroid use	36 (7.5)
Chemotherapy	73 (15.3)
Total parenteral nutrition use	92 (19.2)
Premature baby	27 (5.6)
Neutropenia (<500cell/μL)	39 (8.2)
Neutropenia (<1,000cell/μL)	49 (10.3)
Central venous catheter	325 (68.0)
Intensive care	303 (63.4)
Recent surgery (< 30 days)	133 (27.8)
Intravenous drug usage	11 (2.3)
Multiple antibiotics use (>3 classes)	252 (52.7)
Clinical manisfestations, *n* (%)	
Infective endicarditis	2 (0.4%)
Hepatosplenic candidiases	4 (0.8%)
Endophthamitis	1 (0.2%)
Arthritis	1 (0.2%)

Chapter 7: Genetic Relatedness and Epidemiological Typing

7.1 Background and objectives

Nosocomial outbreaks of infectious diseases not only increase morbidity and mortality, but also hamper public confidence in the health care system. The control of hospital infection is considered an important marker of high professional status for both the health care workers and the health care system. Nosocomial infections, therefore, should be recognised in a timely manner, and the root-cause promptly identified and controlled. Molecular typing is a powerful technique to evaluate whether an unusual cluster of infections is related or not. It has been widely employed for investigating bacterial infections such as MRSA, enterococcus, *Clostridium difficile*, norovirus and many others. In appropriate settings, the identification of clonal relatedness allows confirmation of an outbreak and allow appropriate intervention to be undertaken. While this concept is widely accepted in the fields of bacteriology and virology, its application has only been recently introduced into medical mycology. Admittedly, the biology of eukaryotic fungal organisms and their genetic composition is much more complicated than in the case of viruses and bacteria, thus making progress in this field relatively slow.

Usually, *Candida* infections occur as an endogenous infection, where the organism

originated from the patient self, as shown in the pathology and genetic heterogenicity via genotyping. In situations where infection control practices break down, nosocomial spread via HCW has been documented (van Asbeck 2007; Yildirim 2007). Species such as *C. parapsilosis* group1 are more closely related to theses events, and can be recognised via genotyping (Lasker 2006). Outbreaks caused by other species have also been reported (Lasheras 2007, Roilides 2003).

The difficulty is to identify a reproducible genotyping system, which is not too demanding for the laboratory in terms of technical expertise, thus allowing surveillance systems to be introduced. The typing technique should also be highly discriminatory, as many strains (molecular diversity) can be present in the same patient. To be more cost-effective, screening methods are also now being explored for use prior to embarking on genotyping methods. The technique should also be easily transferable, so that the clinical laboratory can adopt the tool to investigate nosocomial infections. This will also raise the awareness of HCWs that fungal infections can be transmitted nosocomially if there is any breach of infection control practices. Fungal infections are not an exception when compared to bacterial or viral infections.

7.2. Study four: Identification of polymorphic regions in the 18S and 25S rRNA genes for epidemiological typing of clinical Candida albicans isolates

7.2.1 Abstract

Candida albicans often presents with life-threatening invasive infections in immunocompromised patients. As these patients are often given broad spectrum antibiotics beforehand, it has also been observed that they often became extensively colonized with *C. albicans*, for example from tracheal aspirates, urinary catheters or wound swabs. Thus, the question often raised as to whether such *C. albicans* colonization, could predate the advent of invasive diseases in these highly susceptible hosts. The 18S and 28S rRNA genes of *C. albicans* have been screened for genetic variations among a collection of 47 isolates in order to identify highly polymorphic regions for epidemiological typing of *C. albicans*. The two genes were each divided into five regions for polymerase chain reaction amplification. Genetic variations were detected by the single stranded DNA conformation polymorphism techniques. Genetic variations were observed in three out of the ten regions studied. A combined total of twenty patterns were observed among 47 isolates. Such techniques can

facilitate the investigation of genetic relatedness of the organisms. The patterns observed can provide a basis for understanding the acquisition and development of C. *albicans* colonization and invasion.

7.2.2 Methods

The 18S and 25S rRNA genes of *C. albicans* were screened for genetic variations by ribotyping with PCR followed by single-strand conformation polymorphism (SSCP). Primers were designed to amplify 18S and 25S rRNA published sequences from the Genbank (table 7.1). 18S rRNA was screened by 5 pairs of primers which target the whole sequence in 5 regions. 25S rRNA was screened by 11 pairs of primers. Amplicons were analysed for the presence of highly polymorphic regions. Three sets of primers (18S-5F/5R, 25S-3F/3R and 25S-4F/4R) which resulted in the most variations were chosen for ribotyping. The SSCP obtained were numerically encoded as shown in figure 7.1.

Forty-six randomly selected clinical isolates were collected over a 3 year period. Isolates were stored in glycerol broth at -70°C before usage. *C. albicans* ATCC 90028 were also included. Identity of the organisms was confirmed using Gram's stain morphology, cornmeal morphology, germ tube formation and carbohydrate assimilation tests. A single colony of the organisms was picked up after 48hr incubation on Sabouraud agar and suspended in 200mL lysis buffer (100mM Tris-HCl, pH 7.5, SDS 0.5% w/v, 30mM EDTA). It was then incubated for 15 minutes at 100°C. 100ml of 2.5M potassium acetate was then mixed with it and stored on ice for 60 min before centrifuged at 12000rpm for 5 min. DNA was precipitated with an equal volume of

isopropanol, washed with 0.5ml of 70% ethanol and air-dried. PCR was performed in a total reaction volume of 50ul consisting of 1X PCR buffer, 2.5mM $MgCl_2$, 10mM dNTP mix, 1.25U of Taq polymerase, 25uM(each) of the primers 18S-5F/5R, 25S-3F/3R and 25S-4F/4R,1ul (1 to 5ng) of DNA template. PCR was carried out using the following conditions: initial denaturation at 95 °C for 5 min; 35 cycles of denaturation (94 °C for 1 min), annealing (50 °C for 2 min), and extension (72 °C for 3 min); and a final extension step at 72 °C for 5 min. A negative control was performed with each run by replacing the template DNA with sterile water in the PCR mixture. The SSCP gel was run at 10 °C at 600V, 50mA and 30w for 4 hr. Gels were stained with DNA silver staining Kit. Isolates were tested thrice.

7.2.3 Results

The 3 primer sets 18S-5F/5R, 25S-3F/3R and 25S-4F/4R yielded 3, 3, and 8 SSCP patterns (figure 7.1). A combined 20 patterns were observed among the 47 isolates. Twenty-five isolates were highly polymorphic genetically with $\leq$ 2 occurrences. However 16 of them demonstrated the 2-1-3 PCR-SSCP pattern (table 7.2).

7.2.4 Discussion

Outbreaks of *Candida* organisms have been recognized recently. These had involved heart surgery, sternal wound infections and neonatal units (Masala 2003; Pertowski 1995; Reissa 2008). The recognition of outbreak requires a high index of suspicion by the HCW. It is also important to confirm the outbreak by molecular typing techniques.

The ribosomal genes are highly conserved in microbial organisms. Little variation exists in these genes if the organisms are of the same strain. However, more variations can be observed between different strains. This served as a useful basis for molecular typing techniques. To reveal and compare the differences of these ribosomal genes, many molecular methods can be employed. Studies have used restriction enzymes digestion to produce fragments of the digested rRNA for fingerprinting purpose (Doebbeling 1991). Other methods such as sequencing can also reveal nucleotide differences (Muller 1999). In the current study, we have aimed to explore a method to ribotype the *C. albicans* among a collection of clinical isolates.

The use of SSCP will enable one single nucleotide difference to stand out, due to the conformational difference. While the isolates collected are not clinical related, consistent pattern is observed with 16 isolates (demonstrating the combined type of 2-1-3). As this cluster included the types strain *C. albicans* ATCC 90028 as well, it is

possible that regions primed are still much conserved, or certain persisting strain is predominant in our locality. Further refinement of the methods, and exploration into genotyping strains isolated from in various population / hospitals / localities, may clarify the issue. The advantage with PCR-SSCP is that it is relatively rapid, results are available within 2 working days. So it would be useful as an initial screening method. However, it is not an automated method, and requires dedicated and designated personnel for the whole experimental work. On the other hand, the technique is not extremely demanding and should be readily transferable between laboratories. With the completion of the *C. albicans* genome, the genome can now be further scrutinized for functional or pathological gene fragments. These genes may act as targets for further development of sensitive typing methods.

Table 7.1. Primers used to screen for genetic variations in 18S and 25S rRNA genes of *C. albicans*.

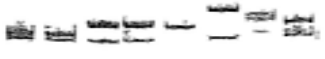
Gene*	Primers	Region		Nucleotide Sequence	Size (bp)
18S rRNA	18S-1F	1	Forward	5'-AACCGTGGTAATTCTAGAGCT-3'	150
	18S-1R		Reverse	5'-AATGAACCATCGCCAGCACA-3'	
	18S-2F	2	Forward	5'-GCCTTCGGGCTCTTTGATGA-3'	330
	18S-2R		Reverse	5'-ACTTGCCCTCCAATTGTTCCT-3'	
	18S-3F	3	Forward	5'-ACACGGGGAGGTAGTGACAAT-3'	485
	18S-3R		Reverse	5'-TGGTAAATGCTTTCGCAGTAG-3'	
	18S-4F	4	Forward	5'-GGTTTCCTAGGACCATCGTAA-3'	535
	18S-4R		Reverse	5'-TATACCAGCAAATGCTAGCAGC-3'	
	18S-5F	5	Forward	5'-GTGCATGGCCGTTCTTAGTT-3'	469
	18S-5R		Reverse	5'-ACCAAGTTTGACCAGCTTCTC-3'	
25S rRNA	25S-1F	1	Forward	5'-TATCAACTTGTCACACCAGAT-3'	124
	25S-1R		Reverse	5'-CTACTGAGGCAATCCCTGTT-3'	
	25S-2F	2	Forward	5'-CAATAAGCGGAGGAAAAGAAACC-3'	215
	25S-2R		Reverse	5'-AGGAACTTTACACAGACCCG-3'	
	25S-3F	3	Forward	5'-CCTTGGAACAGGACGTCACA-3'	330
	25S-3R		Reverse	5'-CCATTATCCTGCCGCTCCAA-3'	
	25S-4F	4	Forward	5'-TGGTATTTTGCATGCTGCTC-3'	410
	25S-4R		Reverse	5'-CAGAGTTTCCTCTGGCTTCAC-3'	
	25S-5F	5	Forward	5'-TGTGTTCGGATGGATTTGAGT-3'	560
	25S-5R		Reverse	5'-ACGCTTGAGCGCCATCCATT-3'	
	25S-6F	6	Forward	5'-TCCGCTAAGGAGTGTGTAACAAC-3'	149
	25S-6R		Reverse	5'-GCAAAGGCTTCGTCACTGACC-3'	
	25S-7F	7	Forward	5'-GCTACTTATACTTCACCGTG-3'	319
	25S-7R		Reverse	5'-TACGTTGCCGTGAAGAATCC-3'	
	25S-8F	8	Forward	5'-GTTAGTCGATCCTAAGAGATGG-3'	440
	25S-8R		Reverse	5'-CCAAGACACCCGATCCTTAG-3'	
	25S-9F	9	Forward	5'-TCTCCAAGGTTAACAGCCTCT-3'	549
	25S-9R		Reverse	5'-GTCAAGCTCAACAGGGTCTT-3'	
	25S-10F	10	Forward	5'-GTGACGCGCATGAATGGATTA-3'	552
	25S-10R		Reverse	5'-AGCAATGTCGCTATGAACGCT-3'	
	25S-11F	11	Forward	5'-AAGTGTGGCCTATCGATCCT-3'	659
	25S-11R		Reverse	5'-ACAAGGCTACTCTACTGCTTAC-3'	

* Sequences based on GenBank assession number: AF114470 (18S rRNA) and X70659 (25S rRNA)

Table 7.2. Ribotyping results of 47 *C. albicans* using PCR-SSCP with the primers: 18S-5F/5R, 25S-3F/3R and 25S-4F/4R.

Strain	Specimen type	Pattern from PCR-SSCP (numeric code)			Combined ribotypes	Frequency
		18S-5F/5R	25S-3F/3R	25S-4F/4R		
RB1	Mid-stream urine	1	1	1		
RB2	Catheter urine	1	1	1	1-1-1	3
RB3	Catheter urine	1	1	1		
RB4	Tracheal aspirate	1	1	8	1-1-8	2
RB5	Catheter urine	1	1	8		
RB6	Mid-stream urine	1	2	6	1-2-6	1
RB7	Catheter urine	2	1	1		
RB8	Tracheal aspirate	2	1	1	2-1-1	3
RB9	Mid-stream urine	2	1	1		
RB10	Bag urine	2	1	1	2-1-1	1
RB11	Tracheal aspirate	2	1	3		
RB12	Tracheal aspirate	2	1	3		
RB13	Pleural aspirate	2	1	3		
RB14	Vaginal swab	2	1	3		
RB15	Suprapubic urine	2	1	3		
RB16	Mid-stream urine	2	1	3		
RB17	Catheter urine	2	1	3		
RB18	Suprapubic urine	2	1	3	2-1-3	16
RB19	Mid-stream urine	2	1	3		
RB20	Blood culture	2	1	3		
RB21	Mid-stream urine	2	1	3		
RB22	Blood culture	2	1	3		
RB23	Mid-stream urine	2	1	3		
RB24	Mid-stream urine	2	1	3		
RB25	Vaginal swab	2	1	3		
RB26	ATCC 90028	2	1	3		
RB27	Pleural aspirate	2	1	4	2-1-4	1
RB28	Pleural aspirate	2	1	5	2-1-5	2
RB29	Pleural aspirate	2	1	5		
RB30	Vaginal swab	2	1	8	21-18	1
RB31	Sputum	2	3	3	2-3-3	2
RB32	Mid-stream urine	2	3	3		
RB33	Mid-stream urine	2	3	4	2-3-4	1
RB34	Mid-stream urine	2	3	5	2-3-5	2
RB35	Mid-stream urine	2	3	5		
RB36	Catheter urine	2	3	7	2-3-7	1
RB37	Mid-stream urine	2	3	8	2-3-8	1
RB38	Sputum	3	1	1		
RB39	Sputum	3	1	1	3-1-1	4
RB40	Blood culture	3	1	1		
RB41	Mid-stream	3	1	1		
RB42	Catheter urine	3	1	5	3-1-5	2
RB43	Mid-stream urine	3	1	5		
RB44	Mid-stream urine	3	1	8	3-1-8	1
RB45	Mid-stream urine	3	2	6	3-2-6	1
RB46	Blood culture	3	3	3	3-3-3	1
RB47	Catheter urine	3	3	4	3-3-4	1

Figure 7.1. Representative SSCP pattern (from triplicate experiments) of *C. albicans* by the three different primer sets (18S-5F/5R) targeting selected regions of 18S rRNA and 25S rRNA (25S-3F/3R, 25S-4F4R). Numerical codes were given to denote different banding patterns.

Primer sets	**18S-5F/5R**			**25S-3F/3R**			**25S-4F/4R**							
Numerical code	1	2	3	1	2	3	1	2	3	4	5	6	7	8
SSCP pattern														

7.3 Study five: Highly discriminating and reproducible typing of Candida albicans by a novel low-stringency random amplification of polymorphic DNA (RAPD) approach

7.3.1 Abstract

A novel approach of random amplification of polymorphic DNA (RAPD) analysis was devised in this study. It is then used to assess the genetic relatedness of a collection of 130 *C. albicans* clinical isolates. The method utilized a specially designed PCR protocol in which a range of annealing temperatures (40°C – 50°C) in each amplification cycle greatly enhanced the efficiency of producing a RAPD profile through reduction of primer-target annealing stringency during PCR. This amplification strategy provided optimal annealing temperatures for all potential target sites so as to maximize the number of PCR products to be generated in a RAPD reaction, the patterns of which are subsequently depicted by polyacrylamide gel electrophoresis and silver staining. Through separate and repeated analyses with different RAPD primer sets, this typing method was found to yield a highly unique and reproducible RAPD banding pattern for each isolate, thus providing extremely good resolution for delineating genetic relatedness and possible

routes of transmission of clinical *C. albicans* isolates. This low-stringency, high-resolution RAPD approach is likely to be applicable not only to epidemiological typing, but also to species identification of various clinically important microorganisms, especially at times of infectious disease outbreaks.

7.3.2 Methods

7.3.2.1 Isolates

A total of 43 randomly chosen, mostly non-identical *C. albicans* strains, isolated from different body sites of 38 patients with *Candida* sepsis/infections within a four year period (1999-2002), were included in this study as a pilot run (table 7.3). Repeated isolates were obtained from four of the 38 patients (P1, P3, P10 and P11). All isolates were grown and purified in Sabouraud-dextrose agar and identified as *C. albicans* using the CHROM-agar Candida medium (Giusiano 1998), followed by confirmation test with the API 20C system (bioMérieux S.A., Montalieu Vercieu, France). The type strain *C. albicans* ATCC 90028 strain and *C. dubliniensis* ATCC MYA-646 were also included as control. A SSCP approach, in which nucleotide polymorphism in a 260 bp fragment in the 28S rRNA of Candida species is depicted (Hui 2000), was used to confirm that all

strains tested in this study were *C. albicans*. Another cohort (2007 – 2009) of 54 isolates from candidaemia and 33 non-candidaemic isolates was then selected for analysis by the developed RAPD methods.

7.3.2.2 Low-stringency-random amplification of polymorphic DNA (RAPD) analysis

Ten isolates, randomly chosen from the first cohort of 44 strains, were subjected to an initial screening for the most desirable RAPD primer sets on the basis of the optimal number of discernable bands produced, as well as their resolution and reproducibility. A total of 10 primer sets, each comprising three arbitrarily designed primers, were utilized in the optimization tests with the 10 strains. Prior to analyses by RAPD, DNA was extracted from a loopful of overnight culture of *C. albicans* cells by boiling. Briefly, the cells were suspended in 200μL of TE buffer (100mM Tris-HCl, 10mM EDTA, pH8.0). This cell suspension was incubated at 100°C for 15 minutes and cooled down to room temperature. 1μL of this suspension, containing the yeast DNA, was used as DNA template in subsequent RAPD reactions. A primer set containing three randomly designed RAPD primers, namely F2, was used for RAPD analysis of the 43 *C. albicans* isolates. These were chosen after exhaustive screening with 10 similar sets of arbitrarily

designed RAPD primers, on the basis of the criterion of producing a desirable number of discernable amplification bands upon RAPD reactions. The nucleotide sequences of the F2 primers are as follows: (A) 5'GCAGCAGCCG3', (B) 5'ACCGCCTCCC3', (C) 5'GCAGCTCCGG3'. Each RAPD reaction contained 5μL of 10X reaction buffer, 5U of Taq DNA polymerase (Qiagen, Hilden, Germany), 50μL of each RAPD primer and 1μL of DNA template. The reaction volume was adjusted to 50μL with distilled water. The mixture was then heated to 95°C for 15 minutes, followed by 40 amplification cycles, each of which consisted of 1 minute at 95°C, 2 minutes at every 1°C increment in the temperature range of 40°C through 50°C, and 3 minutes at 72°C. These were followed by a final polymerization step at 72°C for 5 minutes. RAPD was performed with two different primer combinations: (1) A, B and C, (2) B and C. A negative control containing double-distilled water was included in all RAPD reactions to gauge whether self-primed amplification products were generated.

7.3.2.3 Polyacrylamide gel electrophoresis (PAGE)

RAPD reaction products were analysed by polyacrylamide gel electrophoresis according to the protocol of the ExcelGel DNA analysis kit (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK), followed by silver staining using the PlusOne

Silver Staining Kit (Amersham Biosciences). Isolates exhibiting similar RAPD patterns were re-analysed alongside each other to determine if their patterns were identical. Amplification bands that appeared in the negative control were excluded from all analyses.

7.3.2.4 Cluster Analysis

The relatedness of the RAPD patterns of the tested isolates and the ATCC control strain was analysed by the Dice coefficient method (1) using the BioNumerics program (Applied Maths, Austin, Texas, USA). All discernable bands, except those which appeared in the negative control, were included in the analysis. The results showed the percentage similarity of banding patterns and hence the pattern of clustering among the isolates. Strains with a percentage similarity of 80% or higher were regarded as being in one cluster, in which the strains were likely to be genetically related to each other.

7.3.3 Results

A total of 130 strains of *C. albicans*, including the *C. albicans* ATCC 90028 and *C. dubliniensis* ATCC MYA-646, and were analysed by the new RAPD method in order to

reveal and compare their genetic relatedness. The range of annealing temperatures tested was initially set at 38°C to 52°C. The use of such a wide temperature range for annealing in PCR was found to produce significantly more product bands with excellent reproducibility as compared to the use of a narrow annealing temperature range or only a single annealing temperature, which often failed to produce amplification products. With a wide temperature range, however, a rather long period of time was required to complete the PCR reactions (typically 19 hours). Eventually, one of the primer sets (F2), comprising primers A, B and C, was found to produce the highest number of discernable amplification bands and was chosen for further optimization of the RAPD reactions. For this particular primer set, the best annealing temperature range was eventually found to be 40°C to 50°C. It should also be noted that this primer set nevertheless produced a small number of amplification products in the negative control sample.

The optimized RAPD protocol for F2 was applied to epidemiological typing of the first set of 44 *C. albicans* strains (figure 7.2). Analysis with polyacrylamide gel electrophoresis, followed by silver staining, produced good-resolution RAPD product bands, with each of the test isolates depicting a unique pattern. Further RAPD analysis with each combination of two of the three primers in the primer set F2 was found to

produce different RAPD patterns for each primer combination. However, primer sets A-B and A-C were discontinued from further analysis since the former produced too few amplification bands and the latter produced too many amplification products in the negative control due to self-annealing of the two primers.

The RAPD patterns generated by primer sets A-B-C and B-C were eventually chosen for analysis of the genetic relationship among the 44 *C. albicans* strains collected from year 1999-2002, as well as the subsequent 54 candidaemic and 33 non-candidaemic isolates. A typical RAPD pattern produced by this low-stringency method consisted of more than 6 major amplification bands and a varied number of minor bands, which were also clearly depicted by analysis with polyacrylamide gel electrophoresis and silver staining (Figure 7.2, 7.5, 7.8). RAPD patterns from a given isolate were highly reproducible upon repeated analyses with the chosen primer sets, despite the fact that such RAPD patterns were extremely sensitive to the range of annealing temperatures used and the design of RAPD primers.

Overall analysis of RAPD patterns produced by the two chosen primer sets (A-B-C; B-C) revealed that the clinical isolates from different patients were largely non-identical,

with none exhibiting a RAPD profile identical to that of the *C. albicans* ATCC 90028 strain. The wide variety of RAPD patterns produced also suggested that few major clones associated with a particular site or period of isolation were observed. The genetic relatedness of these isolates was analysed by the BioNumerics programme (Applied Maths), which helped depict genetic clusters among the isolates according to the levels of similarity among the RAPD banding patterns. At a similarity level of 80% or higher, a total of 7 and 8 clusters were identified for primer sets A-B-C and B-C respectively from the first cohort of isolates collected from 1999-2002. (table 7.3, figures 7.3 and 7.4). In the analysis of candidaemic isolates, 12 and 8 clusters were identified (tale 7.4, figures 7.6 and 7.7); whereas the non-candidaemic strains revealed 9 and 10 clusters (table 7.5, figures 7.9 and 7.10). Isolates that were shown to be in the same cluster by one primer set were not necessarily also in one cluster for the other primer set. Nevertheless, isolates exhibiting highly similar RAPD profiles in both reactions were observed. Among these, isolates 12 and 13 were both in clusters C2 and C8 respectively for primer sets A-B-C and B-C. These two isolates were in fact collected from the same patient (P10, Table 7.3). Similar findings were observed with isolates 48 and 49, 50 to 52, 62 and 63, 84 and 85, 86 and 87, 103 to 106, 112 to 114, 115 and 116, 117 and 118, 119 to 122, 126 to 127, 128 to 130, 131 to 133 (tables 7.4, 7.5, figures 7.6, 7.7, 7.9 and 7.10). In fact,

these isolates were recovered from the individual patient repeatedly within a short period of time. This suggested that individual patients can carry a particular strain within a given period. On the other hand, isolates 33, 35 and 41, which originated from three different patients, were found to be in the same cluster of RAPD profiles in tests with each of the two primer sets (clusters C3 and C1 respectively for primer sets A-B-C and B-C). Likewise, isolates 36 and 39, 64 and 70, exhibited RAPD profiles of a similarity level of 80% or higher in both reactions, despite the fact that they were isolated from different patients (table 7.3, 7.4, figures 7.3, 7.4, 7.6 and 7.7). Interestingly, isolates that were known to be collected from identical patients were all shown to be in the same cluster according to results of analysis by the primer set A-B-C with the exception of isolates 122 and 123. Closer examination of these two isolates revealed that they were only marginally similar at a level of 83%. The results of primer set B-C, however, were not as consistent.

7.3.4 Discussion

Random amplified polymorphic DNA (RAPD) fingerprinting is a modified PCR process that utilizes one or more arbitrarily-chosen primers to amplify a number of

fragments from a given DNA template and generate a discrete "fingerprint" which can be resolved by gel electrophoresis (Blumberg 1992, Bostock 1993, Cresti 1999). Alterations by as little as a single base in the primer sequence lead to marked alterations in the fingerprints generated with a given template under the test conditions. Likewise, single base alterations in the genomic template DNA may also lead to changes in the RAPD fingerprints. Hence RAPD is extremely sensitive in depicting subtle genetic differences and hence the genetic relatedness between clinical samples, and is particularly useful in epidemiological typing in cases of outbreaks of infectious diseases. The new RAPD approach that was developed in this study was found to greatly improve the reproducibility of this method in generating a complex RAPD profile for the test samples, so that comparison of these profiles may help reveal the genetic relatedness of the test strains involved. Low reproducibility and poor resolution are two major limiting factors in clinical application of RAPD in epidemiological typing. This is due to the fact that RAPD primers are only arbitrarily designed, for which entirely complementary target sequences do not exist within the pool of test genomic DNA in most cases. Hence the amplification processes begin with annealing of an RAPD primer to a partially complementary target sequence. Such process is highly susceptible to the slightest variations of experimental conditions and often generates only a small number of

products, limiting the resolution of the typing process. The new RAPD approach therefore offers a way to overcome these obstacles through the incorporation of an extended annealing process that facilitates annealing of primers to all potential target sites. Such amplification strategy also maximizes the chance of successful annealing and hence subsequent primer extension in each cycle of the amplification process despite experimental variations.

Another feature of our new RAPD approach is the use of a primer set that comprises up to three RAPD primers for amplification. Various combinations of primer sets were tested for their ability to produce a banding pattern that contains a desirable number of discernable amplification bands. Theoretically, several desirable primer sets should be identified upon extensive testing of arbitrary primers and one or more of the most desirable primer sets are eventually chosen for typing purposes. On the other hand, the degree of typing resolution is dependent on the number of primer sets used for typing, as well as the nucleotide sequences of the chosen primers. As such, the new RAPD method represents a simple, reproducible and flexible typing technique for which the discriminatory power may be adjusted through variations of the primer combinations, in addition to the range of annealing temperature used during RAPD reactions. High

resolution product analysis was also made possible by polyacrylamide gel electrophoresis and silver staining. It should be noted that the majority of RAPD amplification products were of relatively high molecular weight (>600bp), and that they might be better resolved with a lower percentage polyacrylamide gel electrophoresis system (such as 6-8% instead of the 12.5% gel currently used).

The results of RAPD typing using two different primer sets depicted several epidemiologically related clusters in which the strains are probably genetically related to a certain extent, although some of them were isolated from different patients (isolates 33, 35, 36, 39, 41 64, 70). Apart from these, the results were consistent with our prior hypothesis that the majority of these isolates had little relationship among each other, as they were isolated from different patients, body sites as well as different periods of time. The fact that most strains exhibited its own unique RAPD profile confirmed that no major clones of *C. albicans* predominated among hospital infections of this common yeast species. Likewise, no major association between RAPD types and specific body sites was evident. It is conceivable, however, that a typing method with lower discriminatory power will likely produce results that suggest the presence of major clones among the test isolates, if such method cannot differentiate the subtle differences among the isolates.

The high-resolution typing efficiency of this new RAPD technique was also demonstrated by the finding that isolates collected from the same patient could have different profiles if more than one primer sets were used (for example, isolates 12, to 14 from patient P10, table 7.3, isolates 48 to 52 from patient P42, table 7.4). These results suggest the possibility that different strains of *C. albicans* are present even within one body site of a patient.

Due to the extreme sensitivity of this technique, the use of purified sample in analysis is of utmost importance so that the RAPD pattern would not be altered as a result of the presence of host DNA or those of contaminating organisms. On the other hand, the typing resolution of the method may be adjusted to avoid the method being over-sensitive so that the RAPD pattern may be affected by the slightest trace of contaminating DNA. As mentioned above, this could be achieved by adjusting the reaction conditions and number of primers and primer sets in the test to produce an 'optimal' RAPD pattern, using a reference collection of isolates whose genetic relatedness has previously been determined by other typing methods. The full potential for clinical applications of this low-stringency RAPD method, among them the possibility to use as a rapid species identification technique during infectious diseases outbreaks,

will only be realized upon extensive evaluation with not only *C. albicans*, but also other important bacterial and viral species. It is anticipated, however, that exhaustive testing is required in each case to identify the most optimal primers for such applications.

Table 7.3. Summary of clinical information and RAPD typing results of 44 *C. albicans* strains from 1999 to 2002.

Isolate/lane number	Specimen type	Isolation date	Patient identity	Clusters exhibiting ≥80% similarity among 44 strains upon RAPD analysis by two primer sets (ABC & BC)*	
				ABC	BC
1	Pleural	9/1999	P1		
2	Pleural	9/1999	P1		
3	Pleural	9/1999	P2		C7
4	Sputum	2/2000	P3	C7	
5	Sputum	2/2000	P3	C7	C6
6	Tracheal	2/2000	P4		C6
7	Tracheal	2/2000	P5		C7
8	Tracheal	3/2000	P6	C1	
9	Blood culture	10/2000	P7		C1
10	Blood culture	9/2000	P8		C1
11	Blood culture	8/2000	P9		C2
12	Suprapubic	3/2001	P10	C2	C8
13	Catheter urine	3/2001	P10	C2	C8
14	Suprapubic	3/2001	P10	C2	C5
15	Mid-stream	3/2001	P11	C1	C4
16	Catheter urine	3/2001	P11	C1	C3
17	Catheter urine	2/2001	P12	C6	
18	Catheter urine	2/2001	P13	C6	
19	Mid-stream	1/2001	P14	C5	
20	Mid-stream	2/2001	P15		
21	Catheter urine	3/2001	P16	C5	
22	Sputum	2/2001	P17		
23	Mid-stream	4/2001	P18		C5
24	Mid-stream	3/2001	P19		
25	Bag urine	2/2001	P20		
26	Catheter urine	2/2001	P21		
27	Mid-stream	3/2001	P22		C1
28	Mid-stream	2/2001	P23		C1
29	Blood culture	2/2001	P24		C3
30	Blood culture	1/2001	P25		C1
31	Blood culture	5/2001	P26		C1
32	Mid-stream	2/2002	P27		C1
33	Mid-stream	3/2002	P28	C3	C1
34	Mid-stream	3/2002	P29		C1
35	Mid-stream	3/2002	P30	C3	C1
36	Mid-stream	3/2002	P31	C4	C1
37	Mid-stream	3/2002	P32		C1
38	Mid-stream	2/2002	P33		C2
39	Mid-stream	2/2002	P34	C4	C1
40	Vaginal swab	1/2002	P35		C1
41	Vaginal swab	1/2002	P36	C3	C1
42	Vaginal swab	1/2002	P37	C3	C4
43	Mid-stream	1/2002	P38		C5
44	*C. albicans* ATCC 90028				

*Isolates belonging to the same cluster were given the same cluster number.

Numberings were based on results of cluster analyses (figures 7.3 and 7.4).

Figure 7.2. RAPD profiles of 44 *C. albicans* isolates generated by two different primer sets: (a) A-B-C, (b) B-C. (Lanes 1 – 44: refer to table 7.3; **Lane –ve**: no-template control).

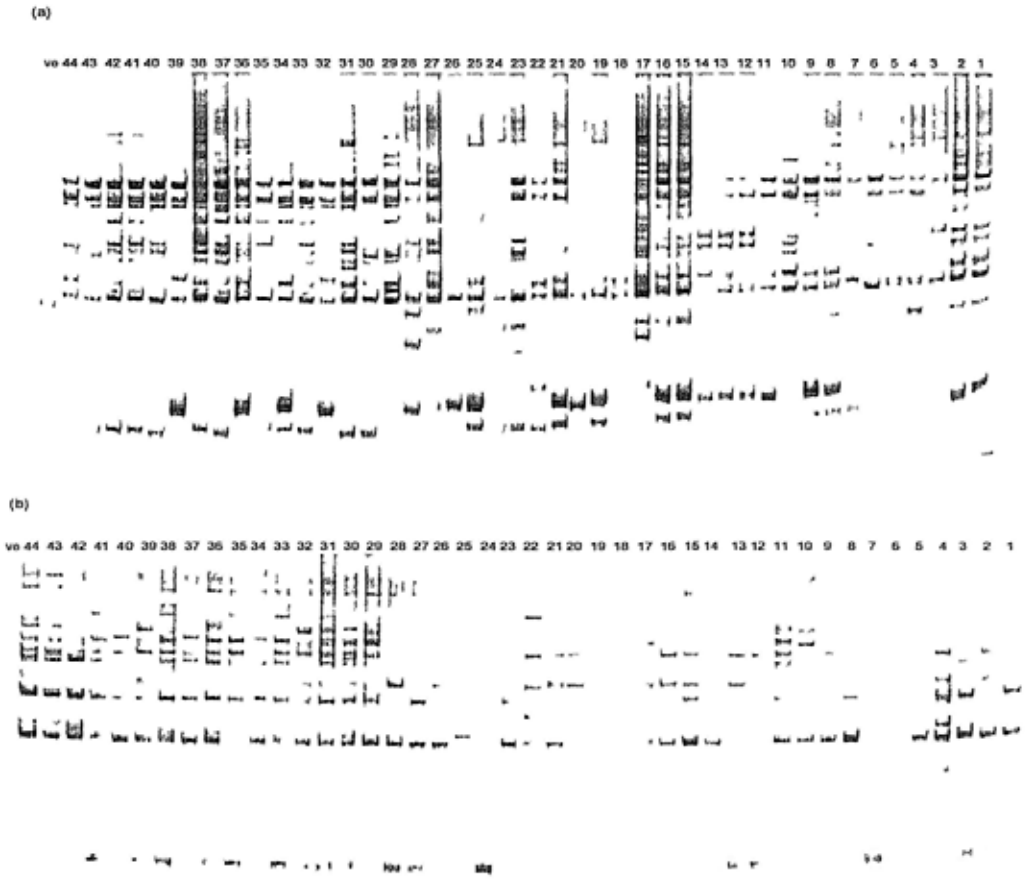


Figure 7.3. Relatedness, in percentage similarity, of RAPD banding patterns of the 44 *C. albicans* isolates generated by primers A-B-C. Isolates with 80% similarity or higher are regarded being in one cluster (**ATCC 90028**, *C. albicans* ATCC 90028; **-ve**, no-template control; **Numbers**: refer to isolate number in table 7.3).

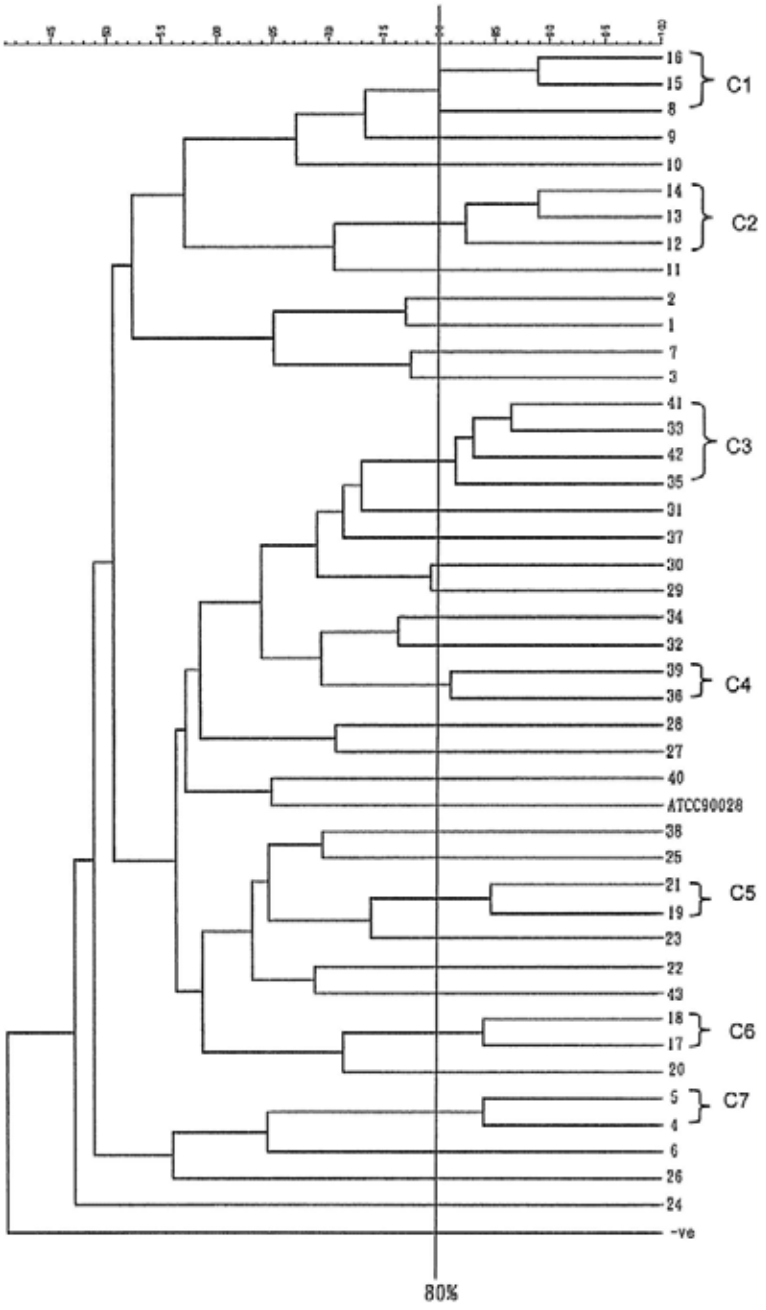


Figure 7.4. Relatedness, in percentage similarity, of RAPD banding patterns of the 44 *C. albicans* isolates generated by primers B-C. Isolates with 80% similarity or higher are regarded being in one cluster (**ATCC 90028**, *Candida albicans* ATCC 90028; **-ve**, no-template control; **Numbers**: refer to isolate number in table 7.3).

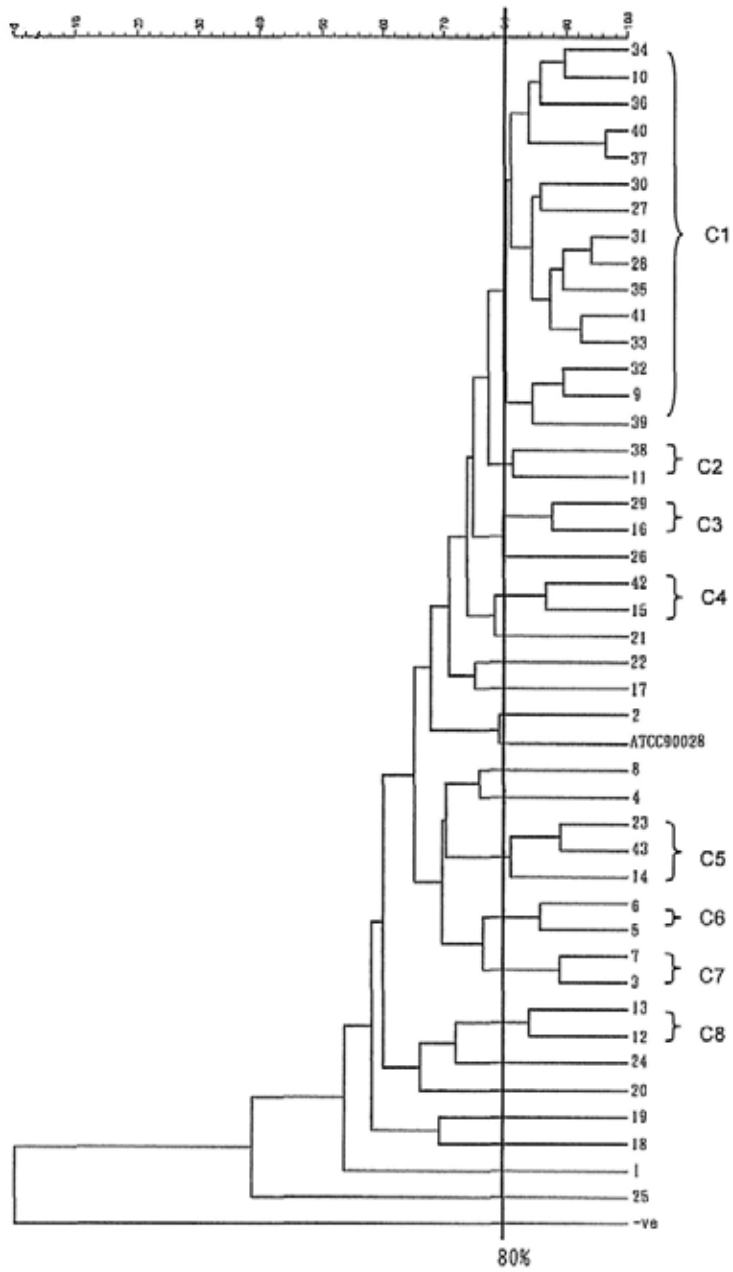
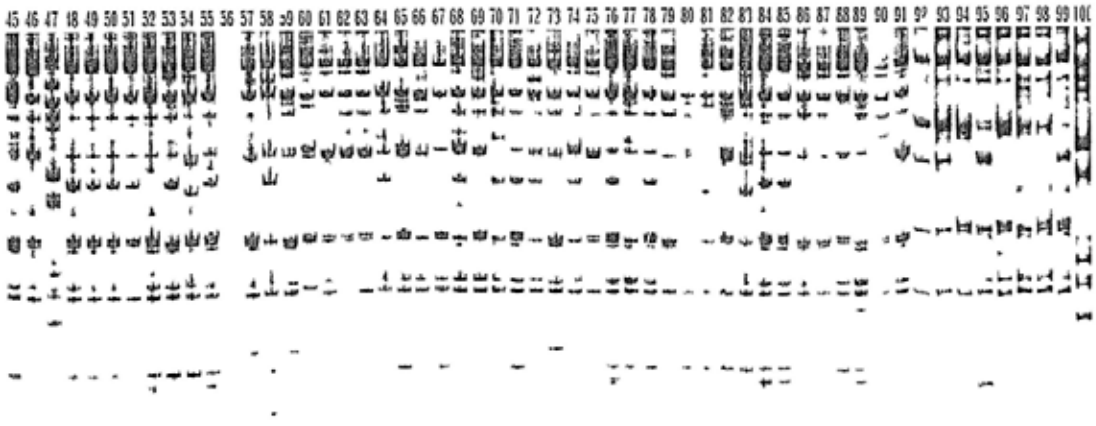


Table 7.4. Summary of clinical information and RAPD typing results of 54 candidaemic *C. albicans* strains.

Strain/lane number	Specimen type	Isolation date	Patient identity	Clusters exhibiting ≥80% similarity upon RAPD analysis by two primer sets (ABC & BC)	
				ABC	BC
45	Blood	6/2008	P39		
46	Blood	7/2008	P40	C6	
47	Blood	7/2008	P41		
48	Blood	3/2008	P42	C5	C1
49	Blood	4/2008	P42	C5	C1
50	Blood	3/2008	P42	C5	C2
51	Blood	3/2008	P42	C5	C2
52	Blood	5/2008	P42	C5	C2
53	Blood	5/2008	P43	C7	
54	Blood	5/2008	P44		
55	Blood	4/2008	P42	C5	C3
56	Blood	3/2008	P42		C3
57	Blood	2/2008	P45	C6	
58	Blood	6/2009	P46	C11	
59	Blood	6/2009	P47	C7	
60	Blood	6/2009	P48		
61	Blood	4/2009	P49	C10	
62	Blood	4/2009	P49	C10	C7
63	Blood	5/2009	P49	C10	C7
64	Blood	8/2009	P50	C12	C8
65	Blood	7/2009	P51		
66	Blood	7/2009	P52	C11	
67	Blood	5/2009	P53		
68	Blood	10/2009	P54		
69	Blood	8/2009	P55		
70	Blood	8/2009	P56	C12	C8
71	Blood	11/2009	P57		
72	Blood	6/2009	P46	C11	
73	Blood	5/2009	P58		
74	Blood	4/2009	P59		
75	Blood	4/2009	P49		
76	Blood	1/2009	P60	C4	C6
77	Blood	12/2009	P61	C9	
78	Blood	12/2009	P62	C4	
79	Blood	3/2009	P63	C8	
80	Blood	7/2009	P51		
81	Blood	6/2009	P64	C9	
82	Blood	7/2009	P51		
83	Blood	6/2009	P64		
84	Blood	3/2009	P65	C3	C6
85	Blood	3/2009	P65	C3	C6
86	Blood	3/2009	P63	C8	C4
87	Blood	3/2009	P63	C8	C4
88	Blood	12/2009	P66		
89	Blood	12/2009	P67		
90	Blood	8/2007	P68		
91	Blood	5/2007	P69		C5
92	Blood	7/2007	P70	C1	
93	Blood	7/2007	P71	C1	
94	Blood	5/2007	P69		C5
95	Blood	9/2007	P72		
96	Blood	5/2007	P69		
97	Blood	7/2007	P73	C2	
98	Blood	7/2007	P73	C2	
99	*C. albicans* ATCC 90028				
100	*C. dubliniensis* ATCC				

Figure 7.5. RAPD profiles of 54 *C. albicans* candidaemic clinical isolates (a) A-B-C, (b) B-C. (Lanes 45 – 100: refer to table 7.4).

(a)



(b)

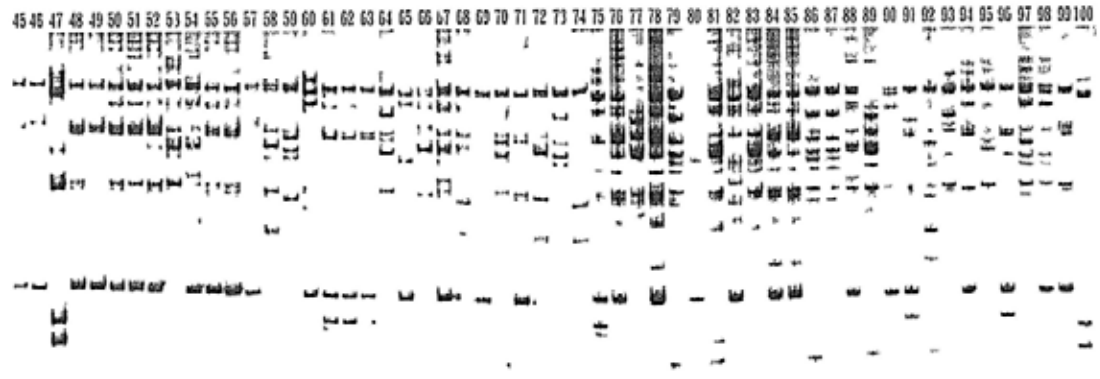


Fig 7.6. Relatedness, in percentage similarity, of RAPD banding patterns of 54 *C. albicans* isolates recovered from blood culture and generated by primers A-B-C. Isolates with 80% similarity or higher are regarded as being in one cluster (**ATCC 90028 CALB**, *C. albicans* ATCC 90028; **MYA-646 CDUB**, *C. dubliniensis* ATCC MYA-646; **Numbers**: refer to isolate number in table 7.4).

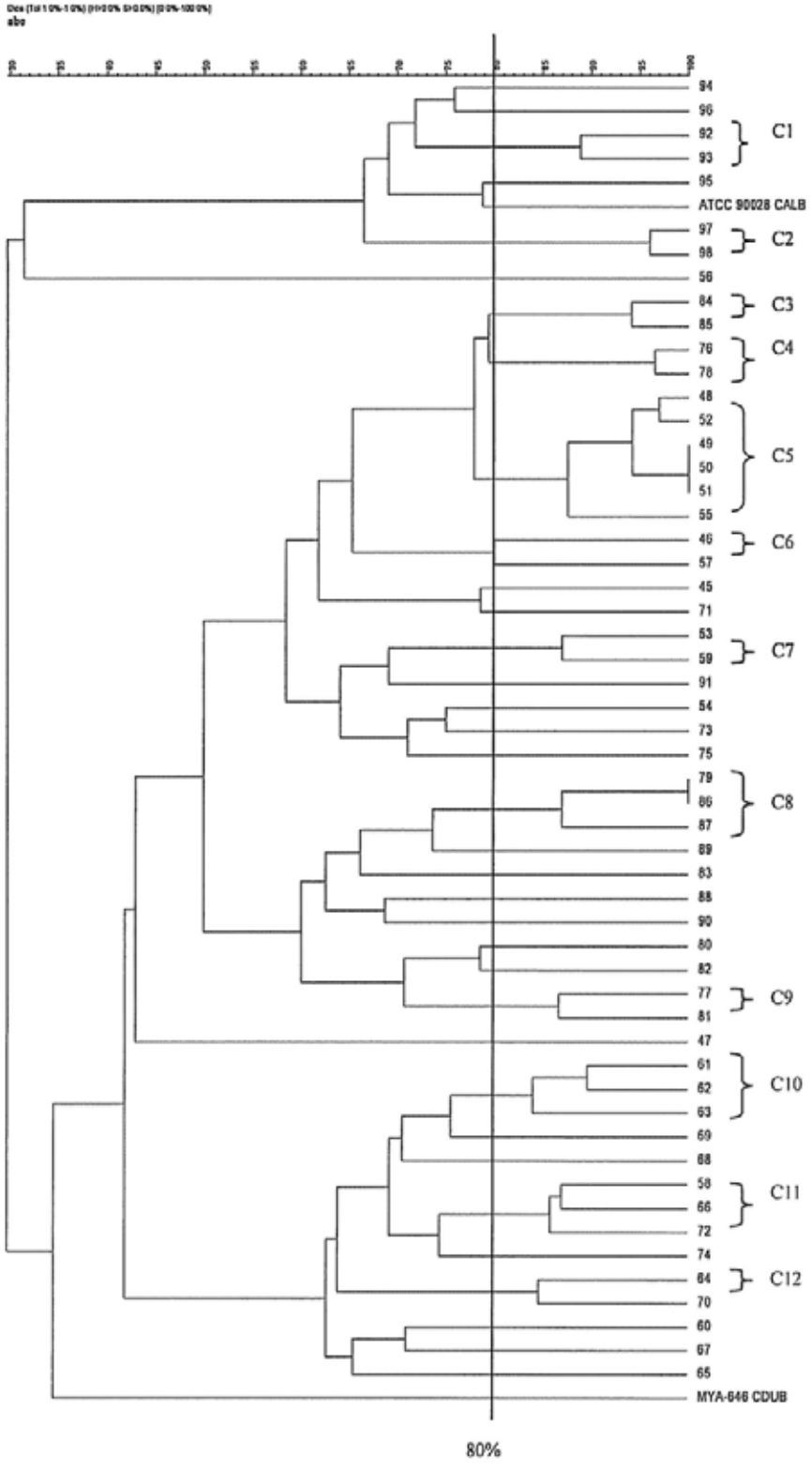


Fig 7.7. Relatedness, in percentage similarity, of RAPD banding patterns of 54 *C. albicans* isolates recovered from blood culture and generated by primers B-C. Isolates with 80% similarity or higher are regarded as being in one cluster (**ATCC 90028 CALB**, *Candida albicans* ATCC 90028; **MYA-646 CDUB**, *Candida dubliniensis* ATCC MYA-646; **Numbers**: refer to isolate number in table 7.4).

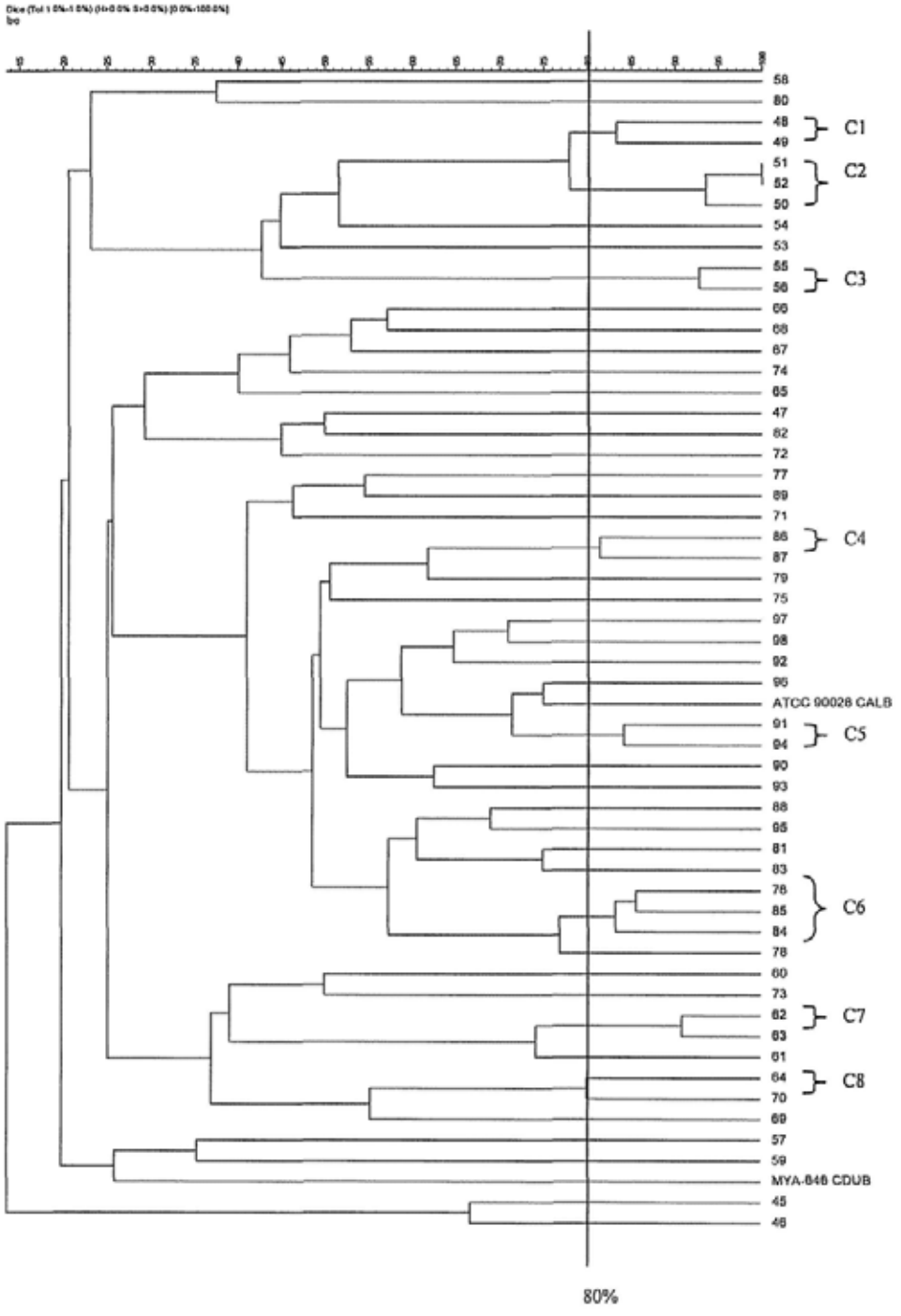
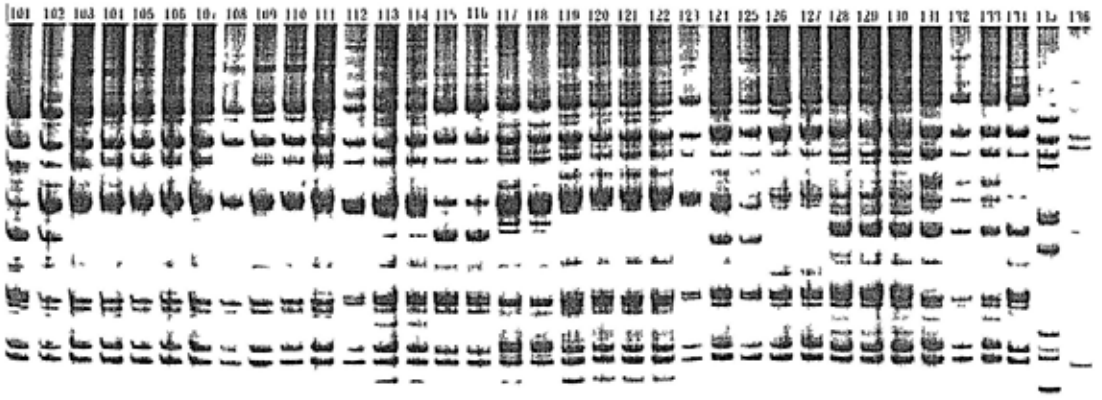


Table 7.5. Summary of clinical information and RAPD typing results of 33 non-candidaemic *C. albicans* strains.

Strain/lane number	Specimen type	Isolation date	Patient identity	Clusters exhibiting ≥80% similarity upon RAPD analysis by two primer sets (ABC & BC)	
				ABC	BC
101	Sputum	2/2009	P74		
102	Catheter urine	2/2009	P74		
103	Tracheal	2/2009	P75	C1	
104	Trachael	2/2009	P75	C1	C8
105	Tracheal	2/2009	P75	C1	C8
106	Tracheal	2/2009	P75	C1	C8
107	Mid-stream	2/2009	P75	C2	C8
108	Mid-stream	2/2009	P75	C2	
109	Catheter urine	2/2009	P75	C2	C9
110	Catheter urine	2/2009	P75		C9
111	Catheter urine	2/2009	P75		C9
112	Sputum	4/2009	P76	C8	C7
113	Sputum	4/2009	P76	C8	C7
114	Sputum	4/2009	P76	C8	C7
115	Sputum	4/2009	P77	C4	C6
116	Sputum	4/2009	P77	C4	C6
117	Mid-stream	4/2009	P78	C7	C5
118	Neprhostomiy	4/2009	P78	C7	C5
119	Mid-stream	7/2009	P79	C3	C4
120	Mid-stream	7/2009	P79	C3	C4
121	Nephrostomy	7/2009	P79	C3	C4
122	Nephtostomy	7/2009	P79	C3	C4
123	Catheter urine	7/2009	P80	C3	
124	Sputum	7/2009	P81	C5	
125	Mid-stream	7/2009	P81	C5	
126	Bronchial	7/2009	P82	C6	C3
127	Bronchial	7/2009	P82	C6	C3
128	Wound swab	10/2009	P83	C9	C1
129	Catheter urine	10/2009	P83	C9	C1
130	Catheter urine	10/2009	P83	C9	C1
131	Tracheal	10/2009	P84	C10	C2
132	Tracheal	10/2009	P84	C10	C2
133	Tracheal	10/2009	P84	C10	C2
134	ATCC 90028 Candida albicans				
135	ATCC MYA-646 Candida				
136	No-template control				

Figure 7.8. RAPD profiles of 33 *C. albicans* non-candidaenic clinical isolates generated by two different primer sets: (a) A-B-C, (b) B-C. (Lanes 101 – 136: refer to table 7.5).

(a)



(b)

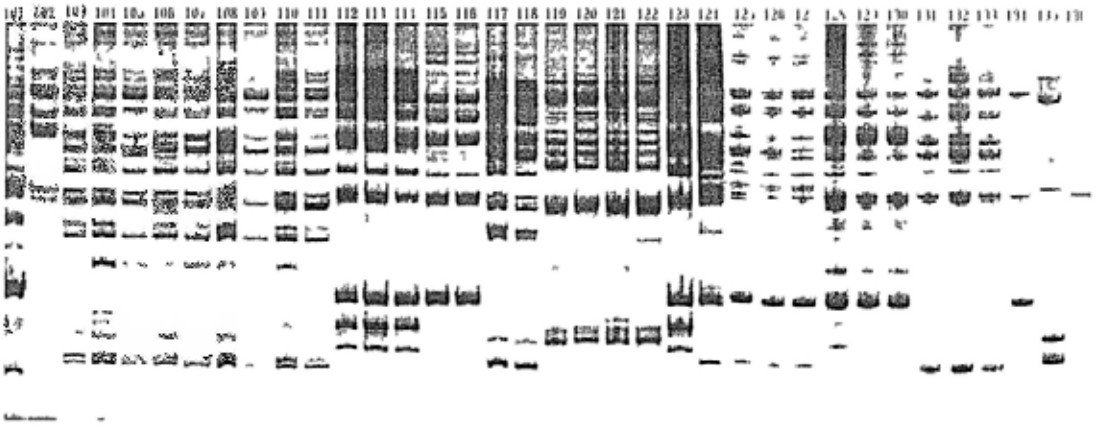


Fig 7.9. Relatedness, in percentage similarity, of RAPD banding patterns of 33 *C. albicans* isolates recovered from different site and generated by primers A-B-C. Isolates with 80% similarity or higher are regarded as being in one cluster (**ATCC 90028 CALB**, *C. albicans* ATCC 90028; **MYA-646 CDUB**, *C. dubliniensis* ATCC MYA-646; **-ve**, no template control).

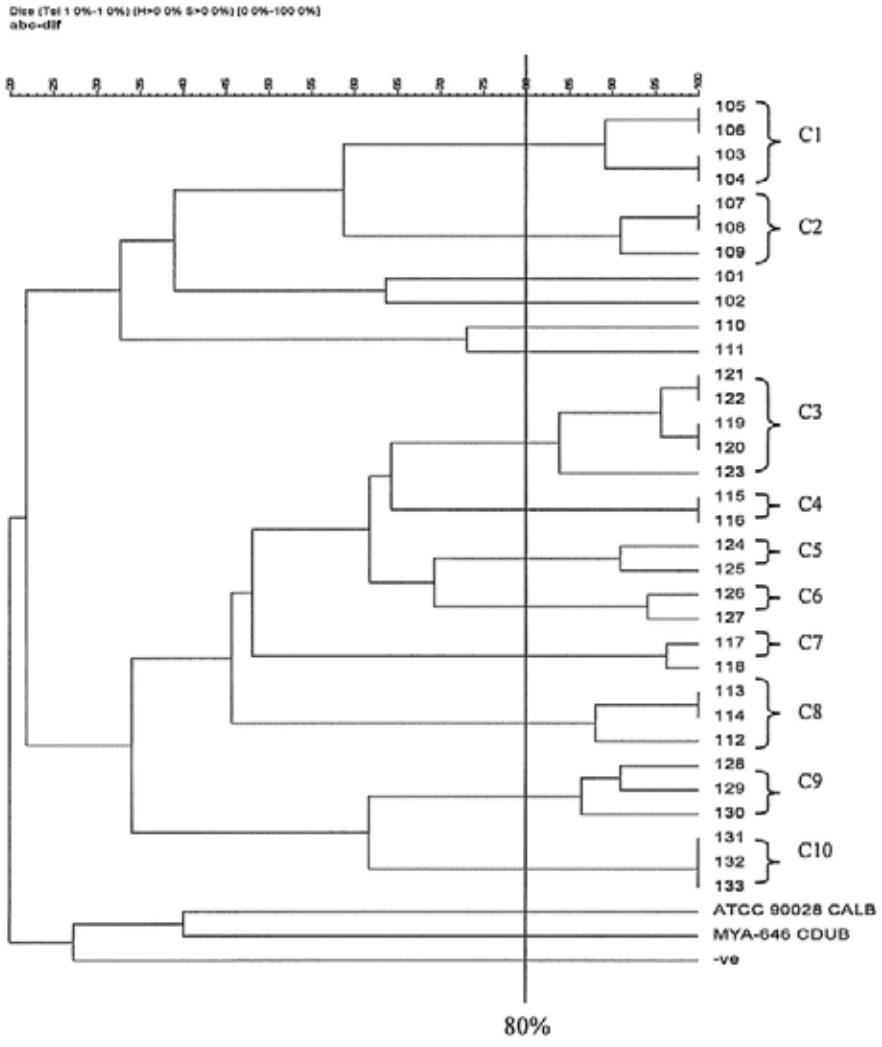
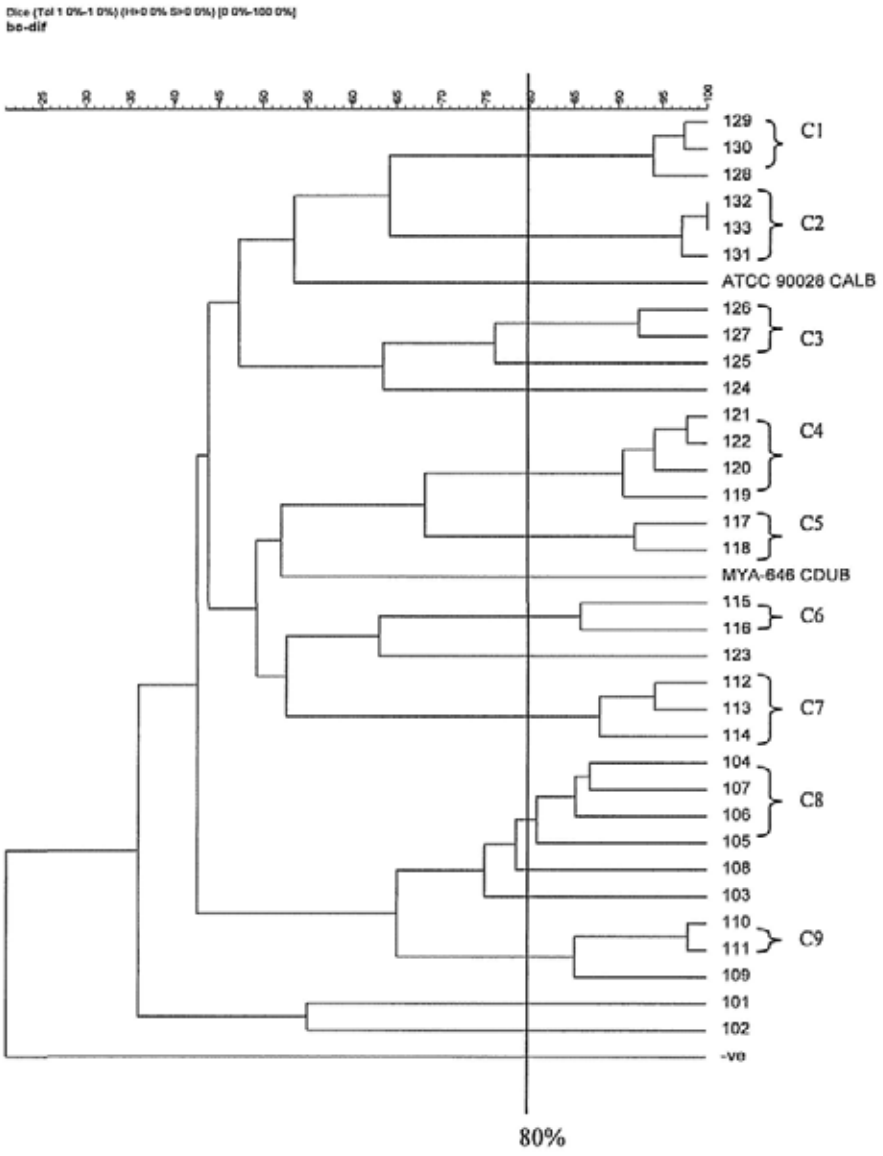


Fig 7.10. Relatedness, in percentage similarity, of RAPD banding patterns of 33 *C. albicans* isolates recovered from different site and generated by primers B-C. Isolates with 80% similarity or higher are regarded as being in one cluster (**ATCC 90028 CALB**, *C. albicans* ATCC 90028; **MYA-646 CDUB**, *C. dubliniensis* ATCC MYA-646; **-ve**, no template control).



Chapter 8: Non-culture based diagnostic methods

8.1 Background and Objectives

Invasive fungal infection has emerged as an important infective complication in immunocompromised patients and is associated with high morbidity and mortality (Jarvis 1995; Verduyn 1999). Clinical diagnosis has often been difficult, and the empirical use of antifungal agents is expensive and not without toxicity (Blumberg 1996; Phillips 1997; Viscoli 1996). Laboratory diagnosis by conventional culture methods is insensitive. Even when a positive culture is obtained, identification of the fungal pathogen is time consuming and difficult.

Among the range of opportunistic fungi, *Candida* species are the most commonly encountered (Farina 1999; Jarvis 1995; Richardson 1998; Warnock 1995). *C. glabrata, C. krusei, C. lusitaniae* and *C. guilliermondii* are of special concern because of their intrinsic resistance to fluconazole or amphotericin B (Abi-Said 1997; Girmenia 1998; Pfaller 1999, 2000). A rapid and specific identification method is of great value to guide the choice of antifungal therapy.

8.2 Study six: Rapid Identification of Medically Important Candida at Species Level using Polymerase Chain Reaction and Single-Strand Conformational Polymorphism

8.2.1 Abstract

Invasive fungal disease has taken a great toll on immunocompromised patients. With the emergence of fluconazole and amphotericin B resistance, the rapid identification of fungi to species level is of clinical relevance in guiding appropriate antifungal therapy. Among these opportunistic fungi, *Candida* species are the most commonly encountered. We had developed a molecular method utilizing single-strand conformational polymorphism (SSCP) to delineate different patterns on a 260-bp amplicon from the 28S rRNA gene from six medically important *Candida* species. The SSCP banding patterns obtained from a total of 52 isolates were sufficiently unique to allow distinction between the species, thus indicated a high level of specificity. This method of PCR-SSCP can provide a simple and specific method for the rapid identification of medically important *Candida* to species level.

8.2.2 Methods

8.2.2.1 Isolates

A total of 52 *Candida* isolates were used in the study. These included six reference ATCC strains and 46 clinical isolates. The ATCC strains were: *C. albicans* (ATCC 24433), *C. glabrata* (ATCC 90030), *C. krusei* (ATCC 6258, 14243), *C. parapsilosis* (ATCC 22019), *C .tropicalis* (ATCC 750) and *C. dubliniensis* (ATCC MYA-646). The clinical isolates were obtained from clinical blood culture isolates from the Prince of Wales Hospital in Hong Kong. They were: eight *C. tropicalis*, three *C. glabrata*, 16 *C. parapsilosis*, one *C. guilliermondii*, two *C. lusitaniae* and 16 *C. albicans*. In addition, two standard strains of *Cryptococcus neoformans* (ATCC 90112, 90113) and one clinical strain of *Trichosporon beigelii* were also included for comparison. The identity of the isolates was confirmed by a combination of microscopic appearance on slide cultures on cornmeal agar, carbohydrate assimilation patterns on API 32C strips (bioMerieux, Missouri, USA) and CHROMagar Candida plates (CHROMagar Company, Paris, France). Yeasts were stored on slants of Sabouraud agar (Oxoid, UK) until use.

8.2.2.2 Extraction of DNA

A single colony of yeast cells was suspended in 200ml lysis buffer (100mM

Tris-HCl, pH 7.5, sodium dodecyl sulphate solution 0.5% w/v, 30mM EDTA). The mixture was incubated at 100°C for 15 minutes, 100ml of 2.5M potassium acetate was then added and mixed. After being stored on ice for 60 min, it was centrifuged at 12000rpm for 5 min. The supernatant was transferred to a new tube. DNA was precipitated with an equal volume of isopropanol, washed with 0.5ml of 70% ethanol, air-dried and subsequently resuspended in 50ml of distilled water. 0.5ml of this DNA solution was utilized as templates in the PCR reactions.

8.2.2.3 PCR

PCR was performed by using the universal fungal primers binding to the conserved regions within the 28S rRNA (Sandhu 1995). The primers used were: 5'-GTGAAATTGTTGAAAGGGAA-3' and 5'-GACTCCTTGGTCCGTGTT-3' (Genset, Singapore). A final PCR reaction volume of 100ml contained 10ml of 10X reaction buffer, 5U Taq polymerase (Phamacia, Uppsala, Sweden), 10mM of each deoxynucleoside triphosphate (Pharmacia), 30mM of each primer and 1ng DNA template. Thirty cycles of amplification were performed in a thermalcycler (9700 series, Perkin-Elmer, Emeryville, Calif.) with an initial denaturation step at 94°C for 5 minutes, annealing step at 55°C for 2 minutes, extension step at 72°C for 3 minutes, and a final extension at 72°C for 5 minutes. Throughout the PCR procedure, precautions were

taken to avoid contamination of the PCR products (Kwok 1989). Negative controls were also included in each test run, with reaction mixture omitting either the primers or the DNA templates. The specificity of the PCR was also performed by using templates prepared from with the following standard strains of bacteria: *Staphylococcus aureus* (ATCC 29213), *Staphylococcus epidermidis* (ATCC 12228), *Enterococcus fecalis* (ATCC 700323) *Enterococcus cloacae* (ATCC19433), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 10031), *Pseudomonas aeruginosa* (ATCC 27853) and *Acinetobacter baumanii* (ATCC 19606).

8.2.2.4 SSCP

PCR products were mixed with equal volumes of a denaturing solution (95% formamide, 0.05% xylene cyanol solution, 0.01% bromophenol blue) and heated at 95°C for 5 min. The mixture was then immediately placed on ice. Four ml of this mixture was loaded onto the 12% ExcelGel (Pharmacia) and subjected to electrophoresis under 600V and a constant gel temperature of 4°C or 15°C for 90 minutes. The SSCP patterns were visualized by silver staining.

8.2.2.5 Simulated blood culture samples with mixed Candida organisms

C. albicans (ATCC 90028) was used to ascertain the detection limit of the PCR.

Overnight culture of the organisms was diluted with BacT/Alert blood culture broth (aerobic) to 10^7cfu/mL. This was then further diluted in serial 10-fold to 10^2cfu/mL (final concentration in blood culture bottle). Cell suspensions were then incubated at 37°C overnight in BacT/Alert blood culture system. Aliquots of 1mL were then taken out for DNA extraction and PCR reactions as described above.

The three most commonly encountered species of *Candida* were chosen to simulate mixed infections. *C. albicans* (ATCC 90028), *C. glabrata* (ATCC 90030) and *C. tropicalis* (ATCC 750) were used. Mixture of 2 or 3 of the *Candida* species, in concentration of 10^4cfu/mL to 10^7cfu/mL were used to approximate the concentration found in candidaemic patients. Aliquots of 1mL were taken after overnight incubation and subjected to PCR-SSCP as described above.

8.2.3 Results

PCR amplicons of 260-bp were obtained from all ATCC strains of yeasts and clinical strains of *C. albicans*, *C. parapsilosis*, *C. glabrata*, *C. tropicalis*, *C. krusei*, *C. guilliermondii*, *C. lusitaniae*, *C. dubliniensis*, *Cryptococcus neoformans*, and *Trichosporon beigelii*. No false amplification was observed with all the eight ATCC bacterial strains nor negative controls, indicating the specificity of the PCR assay.

The SSCP banding patterns were indistinguishable when the experiment was run at 4°C. The banding patterns became unique at 15°C to allow distinction between the eight tested yeasts: *C. albicans*, *C. parapsilosis*, *C. glabrata*, *C. tropicalis*, *C. krusei*, *C. guilliermondii*, *C. lusitaniae*, *C. duliniensis*, *Cryptococcus neoformans* and *Trichosporon beigelii* (figures 8.1 and 8.2). For the following *Candida* species: *C. albicans*, *C. glabrata*, *C. krusei*, *C. tropicalis*, *C. lusitaniae* and *C. parapsilosis*, the SSCP banding patterns obtained among the same species were identical, therefore allowing identification of medically important *Candida* to the species level.

Serial dilution of *C. albicans* (ATCC 90028) in blood culture broth revealed visible PCR bands from 10^7 cfu/mL to 10^4 cfu/mL. At lower concentration, no visible PCR band can be visualized, giving a detection limit of 10^4 cfu/mL (figure 8.3). Combination of *C. albicans* and *C. tropicalis* did not just give rise to the sum of bands as observed from individual organisms, rather, two addition bands were observed, especially at a concentration of 10^6cfu/mL. When *C. albicans* and *C. glabrata* were mixed, again, two more bands were observed. With a mixture of *C. albicans, C. glabrata* and *C. tropicalis*, 4 additional bands were noted (figure 8.4). These additional bands were not present with individual organisms. Difference in inoculum size only affected the intensity of the bands. Therefore, differentiation of the species based on these mixed patterns cannot be

confidently achieved.

8.2.4 Discussion

Conventional methods for identification of fungi have always been difficult, time consuming and technically demanding. In this study, we demonstrated that the 28S-rRNA based PCR-SSCP method was capable of detecting and rapidly identifying a wide range of medically important Candida species. The advantages of this molecular method were simple to perform, specific and practical. Its application can also be extended with modifications for use in diagnosis of fungemia from patients' blood cultures.

At 4°C, there was no distinguishable SSCP pattern among the eight species of yeasts. However, when the temperature was raised to 15°C, unique banding patterns were obtained among the different organisms. Walsh *et al.* had also observed similar phenomenon, in their experiment, the *Aspergillus* species banding were the same at 4°C, but the banding patterns became unique when the temperature was raised to room temperature (Walsh 1995). Since the results of SSCP are dependent on the conditions used, temperature of the experiment needs to be optimized (Nataraj 1999).

The number of isolates for *C. krusei* (n=2), *C. guilliermondii* (n=1) and *C. lusitaniae* (n=2) was limited, as compared with the other *Candida* species. A further study to include a larger number of these isolates would be needed to further ascertain the discriminatory power of the technique. A number of other *Candida* species were not included in the present study, e.g. *C. kefry*. However, the above panel of fungi investigated was the majority of *Candida* isolates seen in our clinical laboratory.

In mixed cultures of *Candida* species, the banding patterns were no longer discreet. Instead, additional bands were present, which cannot be accounted for by individual banding pattern. This suggested that PCR-SSCP method cannot be employed in situations where mixed cultures were present. Fortunately mixed candidaemia is a rare entity. However, it will be prudent to obtain colony culture to ensure pure growth of the organism.

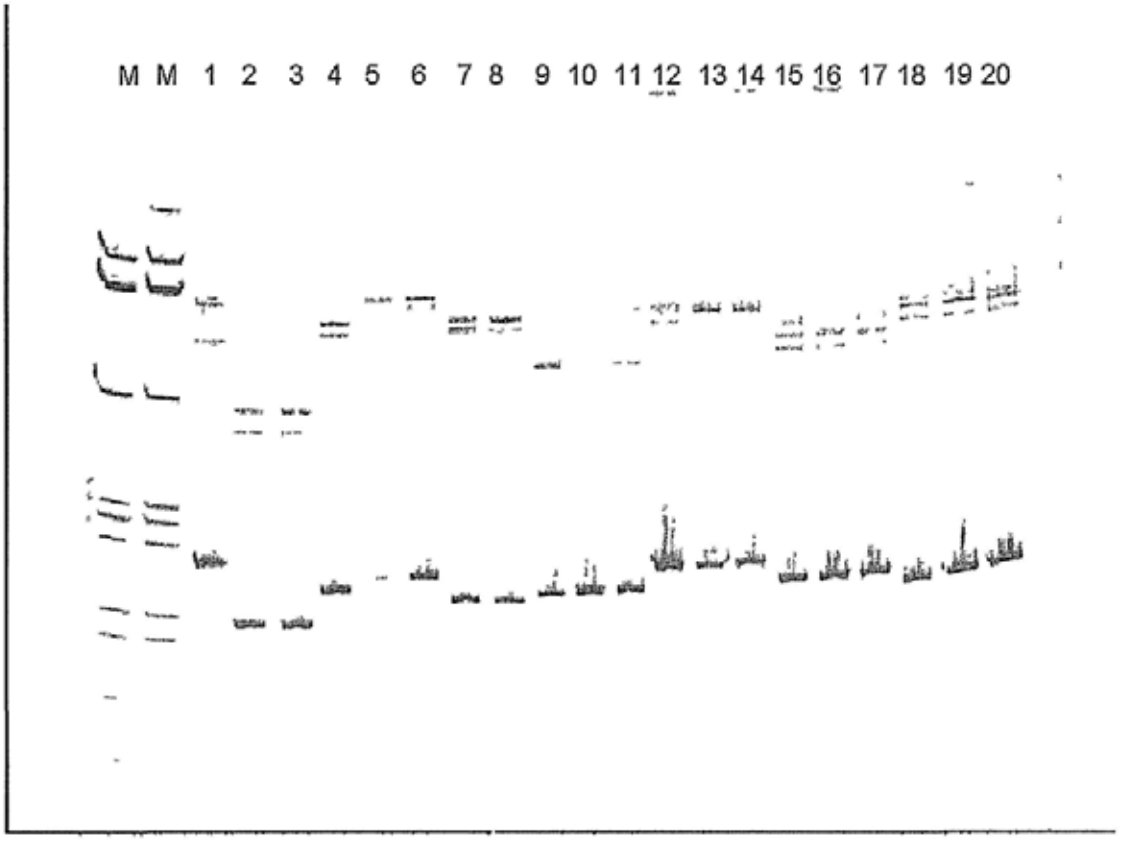
Most clinical laboratory employed germ-tube formation, CHROMagar, cornmeal morphology and carbohydrate assimilation tests to identify *Candida* species. In the event of *C. albicans*, it would be possible to obtain an identity in 1.5 days (after blood culture is flagged positive) if one used the germ tube test as the confirmatory test. For *Candida* non-albicans, it will take at least 2 days (if automated method is used, such as

the Vitek system, bioMerieux Inc). With PCR-SSCP, the steps can be completed within 4 hr after colonial growth. Therefore it could reduce the time to identification for *Candida* non-albicans. The technique itself is not highly demanding and can be readily transfer to any laboratory capable of performing molecular tests. On the other hand, advent of commercially available hybridization technique may further decrease the time to speciation to a few hours after blood culture is flagged positive (Gherna 2009; Reller 2007). The laboratory will have to evaluate and decide on the cost-effectiveness in adopting any of these tests.

In the event of fungemia, the time required to obtain a specific organism identity is critical to patient management. It would be of interest to further explore this method in spiked and direct patient samples. As the PCR is specific for fungal organisms, it would be anticipated that bacterial cause will not result in false positive result.

Therefore, we described a method of PCR-SSCP that is rapid, simple and specific, which can be easily adopted for use in clinical microbiology laboratory for the identification of medically important *Candida*.

Figure 8.1. SSCP patterns of the 260bp amplicons of different yeast strains at 15°C.

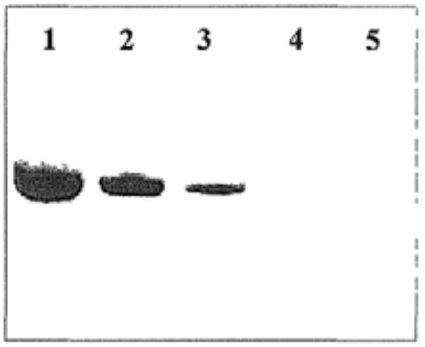


Lane M: *Hin*dIII/ϕ174 marker; **Lane 1** *Trichosporon beigelii*; **Lanes 2-3**: *C. lusitaniae*; **Lane 4**: *C. guilliermondii*; **Lane 5**: *Cryptococcus neoformans* (ATCC 90112); **Lane 6**: *Cryptococcus neoformans* (ATCC 90113); **Lane 7**: *C. krusei* (ATCC 6258); **Lane 8**: *C. krusei* (ATCC 14243); **Lanes 9-10**: *C. tropicalis*; **Lane 11**: *C. tropicalis* (ATCC 750); **Lanes 12-13**: *C. glabrata*; **Lane 14**: *C. glabrata* (ATCC 90030); **Lanes 15-16**: *C. albicans*; **Lane 17**: *C. albicans* (ATCC 24433); **Lanes 18-19**: *C parapsilosis*; **Lane 20**: *C. parapsilosis* (ATCC 22019).

Figure 8.2. SSCP patterns of the 260bp amplocons of 7 ATCC type strains of *Candida* species at 15°C.

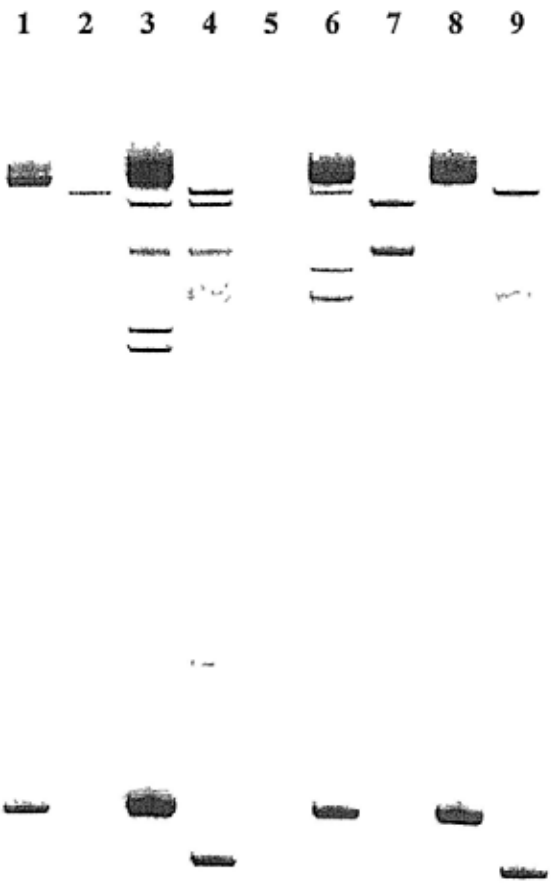
Lane 1: *C. albicans* (ATCC 90028); **Lane 2**: *C. dubliniensis* (ATCC MYA-646); **Lane 3**: *C. glabrata* (ATCC 90030); **Lane 4**: *C. guilliermondii* (ATCC 6260); **Lane 5**: *C krusei* (ATCC 6258); **Lane 6**: *C. parapsilosis* (ATCC 22019); **Lane 7**: *C. tropicalis* (ATCC 750).

Figure 8.3. PCR results of serially diluted *C. albicans* (ATCC 90028) in blood cuture broth.



Lane 1: 10^7 cfu/mL; **Lane 2**: 10^6 cfu/mL; **Lane 3**: 10^5 cfu/mL; **Lane 4**: 10^4 cfu/mL; **Lane 5**: 10^3 cfu/mL.

Figure 8.4. SSCP patterns of mixtures of *Candida* organisms from blood culture broths.



Lane 1: *C. albicans* (10^5 cfu/mL) and *C. glabrata* (10^5 cfu/mL); **Lane 2**: *C. albicans* (10^5 cfu/mL) and *C. tropicalis* (10^5 cfu/mL); **Lane 3**: *C. albicans* (10^6 cfu/mL) and *C. glabrata* (10^6 cfu/mL); **Lane 4**: *C. albicans* (10^6 cfu/mL) and *C. tropicalis* (10^6 cfu/mL); **Lane 5**: *C. albicans* (10^4 cfu/mL), *C. glabrata* (10^5 cfu/mL) and *C. tropicalis* (10^4 cfu/mL); **Lane 6**: *C. albicans* (10^6 cfu/mL), *C. glabrata* (10^7 cfu/mL) and *C. tropicalis* (10^6 cfu/mL); **Lane 7**: *C. albicans* (10^6 cfu/mL); **Lane 8**: *C. glabrata* (10^6 cfu/mL); **Lane 9**: *C. tropicalis* (10^6 cfu/mL)

8.3 Study seven: Development and Application of a Rapid Diagnostic Method for Invasive Candidiasis by the Detection of D-/L-Arabinitol Using Gas Chromatography/Mass Spectrometry.

8.3.1 Abstract

A rapid non-culture-based diagnostic method utilizing D-/L-arabinitol (DA/LA) ratios as a chemical marker of invasive candidiasis was developed and explored. The enantiomers-ratios detection was made possible by the use of gas chromatography coupled with mass spectrometry (GC/MS). The mean DA/LA ratios ± standard deviation (range) in urine (n = 40) and serum (n = 20) were 2.08 ± 0.78 (0.57 to 3.55) and 1.79 ± 0.75 (0.74 to 3.54) respectively from patients without evidence of fungal infection or colonization; 9.91 ± 3.04 (7.24 to 16.27) and 13.58 ± 7.31 (5.57 to 25.88) in urine and serum respectively in patients (n = 7) with culture proven invasive candida infections. The difference in DA/LA ratios between the candidaemic patients and the non-candidaemic patients were statistically significant ($P < 0.01$) in both serum and urine samples. The DA/LA ratios were not significantly affected in patients with oral or vaginal candidiasis and candiduria.

8.3.2 Methods

8.3.2.1 Establishing the linearity of the GC-MS standard

The D-arabinitol standard solutions (Sigma, St. Louis, MO, USA) were serial diluted to known concentrations of 5pg/μL, 50pg/μL, 500pg/μL, 5ng/μL and 50ng/μL which contains fixed amount of 25μ/ml L-arabinitol (Sigma, St. Louis, MO, USA). The samples were then prepared for GC as described below.

8.3.2.2 Type strains of Candida and bacteria

Standard type strains of *Candida* organisms used were *C. albicans* ATCC 24433, *C. tropicalis* ATCC 750, *C. parapsilosis* ATCC 22019, *C. krusei* ATCC 6258 and *C. glabrata* ATCC 90030. *C. kefyr*, *C. lusitaniae*, *C. guilliermondii* and six clinical isolates of *C. krusei* were obtained from our laboratory stock clinical isolates. *Cryptococcus neoformans* ATCC 90113, *Saccharomyces cerevisiae* ATCC 36375 and *Trichosporon beigelii* (clinical stock isolate) were also included. Specificity of the method was tested with the use of commonly encountered blood and urine bacterial isolates. These include type strains of *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 10031, *Pseudomonas aeruginosa* ATCC 10662 and *Acinetobacter*

baumannii ATCC 19606.

8.3.2.3 The production of D-arabinitol by different Candida species

The *Candida* organisms were grown in Sabouraud broths at 37°C for 48 hours and then adjusted to 0.5 McFarland standard. They were then prepared for GC analysis. The type strains of bacteria for specificity testing were placed in 4.0 ml nutrient broth each and incubated at 37°C for overnight. The broths were adjusted to 0.5 McFarland standard and prepared for GC-MS as described below.

8.3.2.4 Preparation of samples for GC

Cultures were first filtered to remove solid materials. Aliquots of 20μL were transferred into 1.0 ml vials and evaporated to dryness under a stream of nitrogen. Trifluoroacetic anhydride (Sigma, St. Louis, MO, USA) and n-hexane (Sigma, St. Louis, MO, USA) at 200μL each were then added. The samples were subsequently heated at 80°C for 10 minutes. After cooling down to room temperature, the samples were dried again under a stream of nitrogen. Finally, 200μL of n-hexane was added, and subsequently analyzed by GC (Larsson 1994).

8.3.2.5 GC-MS detection

The samples were analyzed using an HP5890A gas chromatograph (Hewlett-Packard) equipped with a fused silica capillary column (30 m long, 0.25 mm internal diameter, 0.25μm thick cyclodextrin [Beta-Dex-120; Supelco, Bellefonte, PA, USA]). The column temperature was programmed to rise at 4°C/minute from 70°C to 190°C. The ion source temperature was 210°C. The column was coupled to an HP5972 mass selective detector (Hewlett-Packard). Analysis was performed in the electron impact mode by using selective ion monitoring with an m/z of 519. D-/L-arabinitols were determined from peak areas.

8.3.2.6 Urine samples

Control groups

All patient samples in the control group were non-duplicate. These urine samples were collected in 10 ml aliquots and kept frozen at –20°C until analysis. All urine samples were then prepared as described for GC-MS analysis. Samples were randomly selected and based on information from laboratory request forms specifying oral thrush, vaginal thrush or cystitis together with compatible microbiological cultures.

(A) Sterile urine samples from adult patients attending the out-patient clinic with no known Candida infection or colonization were collected. A total of 40 samples were

obtained.

(B) Urine samples from adult patients attending our out-patient clinic with significant bacteriuria (bacterial count > 10^5CFU/ml) were collected. A total of 40 samples were obtained.

(C) Sterile urine samples from adult patients attending the out-patient clinic with evidence of Candida colonization were collected. These included oral candidiasis (n = 15) and vaginal candidiasis (n = 15).

(D) Urine samples from adult patients attending the out-patient clinic with Candiduria were collected, a total of 10 samples were collected.

(E) Aliquots of 10 ml sterile urine samples obtained from (A) above were spiked with 1 x 10^6 CFU of each *Candida* organisms. These were then incubated at 37°C for 48 hours.

Infected group

Over the time course of one year, urine samples from patients with blood culture proof of invasive *Candida* infections were collected. The criteria for proven cases include positive blood culture together with underlying risk factors, including indwelling central venous catheter, prolonged use of broad spectrum antibiotics, and use of immunosuppresants such as high dose steroids, chemotherapeutic agents, or deep tissue

involvement. These urine samples were collected in 10 ml aliquots on the day of blood culture flagging positive signal and a Gram's stain revealing yeast-like cells. The samples were kept frozen at –20°C until analysis. Patients' demographic data were also obtained on a standard log. Samples from 7 patients with invasive diseases were collected.

8.3.2.7 Serum samples

Control groups

Samples were collected from a separate group of patients whom no urine sample had been collected for this study.

(A) Sterile serum samples from adult patients attending the out-patient clinic with no known Candida infection or colonization were collected. A total of 20 samples were obtained.

(B) Adult bacteraemic patients' sera were collected. A total of 20 serum samples were obtained.

(C) Sterile serum samples obtained in (A) were divided into 1 ml aliquots and spiked with 1 $x10^5$ CFU of each Candida organisms, the samples were then incubated at 37°C for 48 hours.

Infected Group

Over the time course of one year, serum samples from patients with blood culture proof of invasive Candida infections were collected. These serum samples were collected in 3 ml aliquots on the day of blood culture flagging positive signal and a Gram's stain review yeast-like cells. The samples were kept frozen at –20°C until analysis. Patients' demographic data were also obtained on a standard log. Samples from 7 patients with invasive diseases were collected.

Statistical analysis

Descriptive statistics were presented as mean ± standard deviation (range) where appropriate. Differences between means were compared using unpaired two-tailed Student's t test. All P values of <0.05 were considered as significant.

8.3.3 Results

The mass spectrum of trifluoroacetate derivatives of arabinitol was shown in figure 8.5. The spectra were the same for D-and L- arabinitol. In the chromatographic conditions used in this study, D-arabinitol eluted at a relative retention time of 9.04 minutes, followed by L-arabinitol 0.3 minute later (figure 8.6). The calibration curve was

linear.

D-arabinitol can be detected from broth cultures of ATCC type strains of *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*.and clinical isolates of *C. lusitaniae*, *C. guilliermondii* and *C. kefyr* and the six clinical isolates of *C. krusei*. The only *C.* species that did not produce D-arabinitol when tested was *C. glabrata*.

Cryptococcus neoformans, *Saccharomyces cerevisiae* and *Trichosporon beigelii* also did not produced measurable D-arabinitol when tested. All bacterial broth cultures showed no production of D-arabinitol.

The reproducibility of the assays was determined by choosing two samples of urine that showed low and high DA/LA ratios on initial assay (0.57 and 3.55 respectively). This two urine samples were then divided into 10 equal aliquots and each underwent derivatization and GC/MS analysis. The calculated interassay imprecision percentages were 2.4% and 4.1% for low and high urine DA/LA ratios respectively.

Forty adult patients from out-patient clinics without evidence of invasive *Candida* infection nor colonization (group A) had a mean ± standard deviation (range) urine

DA/LA ratio of 2.08 ± 0.78 (0.57 to 3.55). Whereas the results for patients with bacteriuria (group B, n = 40) was 1.94 ± 0.56 (0.76 to 3.18); for patients with concurrent oral candidiasis (group C, n = 15), 1.77 ± 0.60 (0.91 to 3.26); for patients with vaginal candidiasis (group D, n = 15), 1.87 ± 0.52 (0.94 to 3.11); for patients with candiduria without clinical suspicion of invasive disease (group E, n = 10), 2.16 ± 0.68 (1.09 to 3.24) (Table 8.1).

Statistical analysis showed that the difference in DA/LA ratios between control groups of A to E were not significant ($P > 0.05$). Spiking of sterile urine sample with each Candida organisms did not show significant change in DA/LA ratios after incubation of 48 hours.

The candidemic patient group (group H, n = 7) showed urine DA/LA ratios of 9.91 ± 3.04 (7.24 to 16.27). It showed significant difference when compared to each of the control groups A to E ($P < 0.01$).

The reproducibility of the assays was determined by choosing two samples of sera that showed low and high DA/LA ratios on initial assay (0.74 and 3.54 respectively). These two sera samples were then divided into 10 equal aliquots and each underwent

derivatization and GC/MS analysis. The calculated interassay imprecision percentages were 7.45% and 3.4% for low and high urine DA/LA ratios respectively. Twenty adult patients from out-patient clinics without evidence of invasive *Candida* infection nor colonization (group F) had a mean ± standard deviation (range) serum DA/LA ratio of 1.79 ± 0.75 (0.74 to 3.54). Whereas the result for patients with bacteremia (group G, n = 20) was 2.00 ± 0.67 (0.91 to 3.17).

Statistical analysis showed that the difference in DA/LA ratios between control groups of F and G were not significant ($P > 0.05$). Spiking of sterile serum sample with each of the 7 species of *Candida* organisms did not show significant change in DA/LA ratios after incubation of 48 hours.

The candidaemic patient group (group I, n = 7) showed serum DA/LA ratios of 13.58 ± 7.31 (5.57 to 25.88). It showed significant difference when compared to each of the control groups F and G ($P < 0.01$).

Demographic data of the group of candidaemic patients and their causative *Candida* species were shown in Table 8.2. Of the seven candidaemic patients, four were diagnosed to have haemtological malignancy with acute myeloid leukaemia (AML), one

with systemic lupus erythematosus (SLE), one with 50% burns injury and one patient was an intravenous drug addict with *Candida* endocarditis. All four patients with AML had received chemotherapy and suffered from neutropenic fever at the time of diagnosis of candidaemia. They all had central venous catheter in situ. All seven patients received antibiotics prior to the onset of fungaemia. Only one patient survived, who was an intravenous drug abuser with Candida endocarditis, requiring valvular replacement.

8.3.4. Discussion

The use of arabinitol as chemical marker for diagnosis of candidiasis was described as early as in 1979. However, earlier investigators had measured the total amount of arabinitol, without differentiating into enantiomers, thus resulting in insensitive results (Eng 1981; Wells 1983). With the advent of chiral stationary phase columns, the enantiomers could then be separated. Roboz and associates described the normal values of serum DA/LA ratios to be 1.40 ± 0.42 (mean ± S.D.). By taking a value of ≥ 2.24, defined as normal mean ± 2 S.D., he was able to identify 10 out of 12 truly candidemic patients (Roboz 1990, 1992). In 1994, Larsson *et al* explored the method further by measuring DA/LA ratios from urine. He was able to correlate an increase in ratios with patients suffering from disseminated candidiasis. Also, there was no difference in the

mean ratios between healthy subjects' urine and candiduric subjects without disseminated disease (Larsson 1994).

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In the current study, we attempted to measure DA/LA ratios in urine and serum of various patients groups. It is important to establish a baseline ratio in our patient population. Various investigators had reported different mean DA/LA ratios, ranging from 1.40 to 2.50 (Christensson 1997; Lehtonen 1996; Roboz 1992; Sigmundsdottir 2000; Stradomska 2002). We found that in our studied group, the healthy adults' mean serum ratio was 1.79, and the urine ratio was 2.08. These results were similar to the findings of previous investigators.

By defining a cutoff value of mean ratio + 2 S.D. as the upper limit, and compare this value (3.64 and 3.29 for urine and serum ratio respectively) with the findings of the seven culture-proven candidemic patients. The DA/LA ratio would diagnose all of the candidemic patients, but misdiagnose one healthy adult who had a serum DA/LA ratio of 3.54. However, by raising the cutoff value to mean ratio + 3 S.D., none of the normal subjects would be misdiagnosed. Although Roboz and his associates had used mean ratio + 2 S.D. as their cutoff limit, subsequent investigators had mostly employed mean ratio + 3 S.D. as the upper limit of normal (Christennson 1997; Sigmundsdottir 2000;

Stradomska 2002). Although the sensitivity of the assay appeared satisfactory in the present study, the total number of culture-proven candidemic patients was small (n = 7); therefore, more patients could be recruited to validate the sensitivity of the method. Furthermore, whether the method could be employed to diagnose early phase of disease dissemination, provide guidance to the initiation of empirical or pre-emptive therapy, as well as monitoring of patient's response to therapy, would require further prospective study.

We had also demonstrated that superficial candida infections, such as oral and vaginal candidiasis, had no influence on the serum and urine DA/LA ratios. This would suggest that such superficial infections would have no interference with the GC/MS detection method developed in this study. With spiking of serum or urine with *Candida* organisms, again, no significant changes in the ratio was observed after incubation. This may suggest that in vitro spiking of the organisms would not simulate an invasive infective process. This was important as patients who are at high risk of invasive candida infections, are often being prescribed broad-spectrum antibiotics that could easily lead to superficial candida colonization, and also subsequent contamination of collected specimens.

Candiduria, which can be considered as superficial infection or colonization in the absence of symptoms and signs, was also shown to have no interference with the method. However, more work with larger sample size is still needed, as in some patients; the presence of candiduria could be predictive of subsequent invasive disease (Fisher 2000).

As DA/LA ratio measurements were comparable between serum and urine samples, and would not be interfered by superficial colonization nor candiduria, this method would provide a non-invasive mean of diagnosis by sampling urine specimens.

In our panel of *Candida* organisms tested, we were capable of dete cting one more organism than was previously suggested, which was *Candida krusei*. This result was obtained by testing not only the ATCC type strain, but also six clinical isolates. This may be due to the use of more sensitive detection method of selected ion monitoring, rather than the electron capture detector that was previously used (Roboz 1990).

In the present study, the required final injection volume was only 5μL. This suggested that such method could be employed in the neonates who may not tolerate large amount of blood sampling. In clinical practice, small amount of blood obtained from a heel-stab would yield enough sample for DA/LA measurements.

The time required for the preparation of samples and analysis by the GC/MS machinery was also very short and could be completed within one working day. This would provide a rapid diagnostic tool, which is comparable to nucleic detection method.

On the other hand, not all clinical microbiological laboratories are equipped with GC/MS apparatus. The operation of GC/MS equipment requires designated personnel. Moreover, these techniques cannot provide information about the species of the organisms which in turn cannot guide specific antifungal usage. The absence of D-arabinitol from *C. glabrata* also limits the usefulness of this test, as this organism has recently emerged as a major pathogen as well as processing intrinsic azole resistance.

The reason for the lack of D-arabinitol production from *C. glabrata* is still unknown. In *C. albicans*, it had been shown that the organisms utilized D-arabitol dehydrogenase to catalize the production of D-arabitol from D-ribulose (Wong 1993). Null mutant can be constructed by disrupting the corresponding *ard* gene (Wong 1995). Thus it seems possible the *C. glabrata* had no such dehydrogenase, or the gene is suppressed constitutively.

Over the time course of one year, only seven patients had culture e proven systemic candidiasis. They all had risk factors for invasive fungal infection. Such small sample thus can serve as preliminary study on the application of the method. A bigger sample size, as well as the need of a control group with risk factors and superficial *Candida* colonization, would be needed to quantify the influence of each risk factor, the correlation with DA/LA ratio, and possible association with treatment and outcome. It would also be useful for laboratories to compare this chemical based method with molecular based methods in the rapid diagnosis of fungal infections, which will allow a decision to made on what technique is more cost-effective. Our current findings would suggest that this method was easy to use, reproducible, and further application of which could be explored.

Figure 8.5. Mass spectrum of trifluoroacetylated D-arabinitol. The spectra were the same for D-and L- arabinitol. Ions of mass–to-charge ratio (*m/z*) 519 was used for selected ion monitoring.

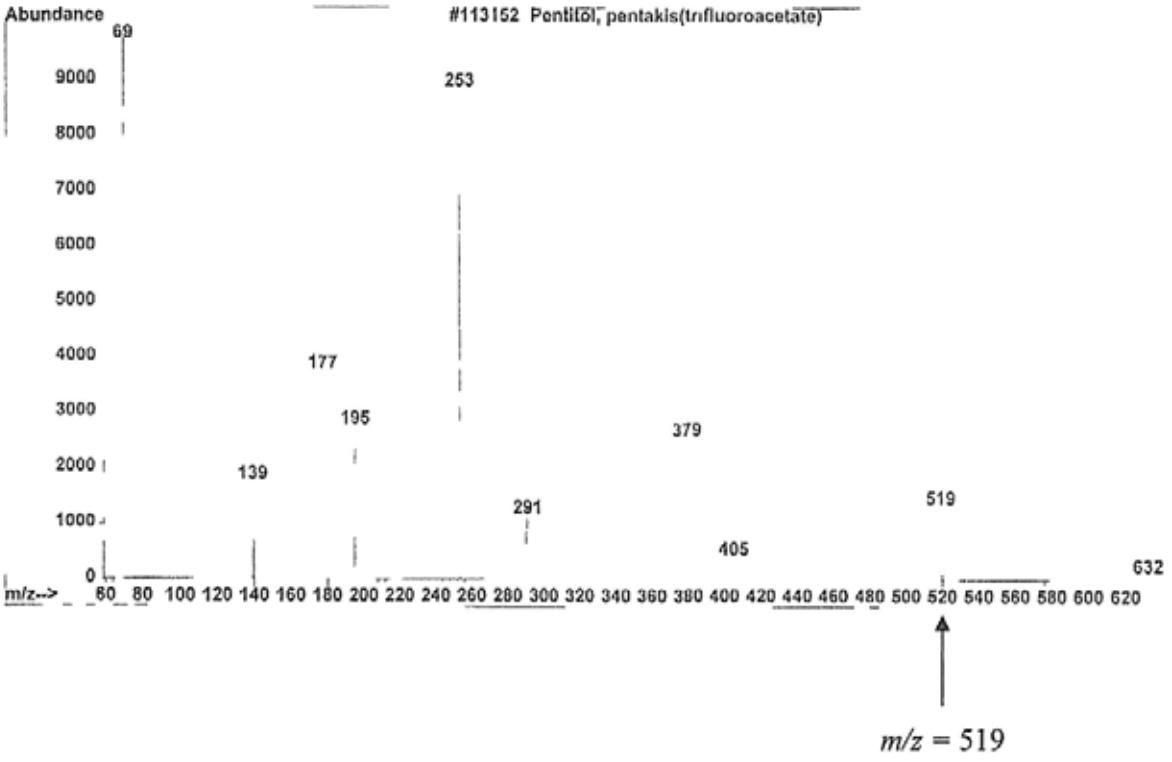


Figure 8.6. Separation of 50µg/ml D- and L-arabinitol by GC-MS with selective ion monitoring at *m/z* 519. D-arabinitol eluted at a relative retention time of 9.04 minutes, followed by L-arabinitol 0.3 minutes later.

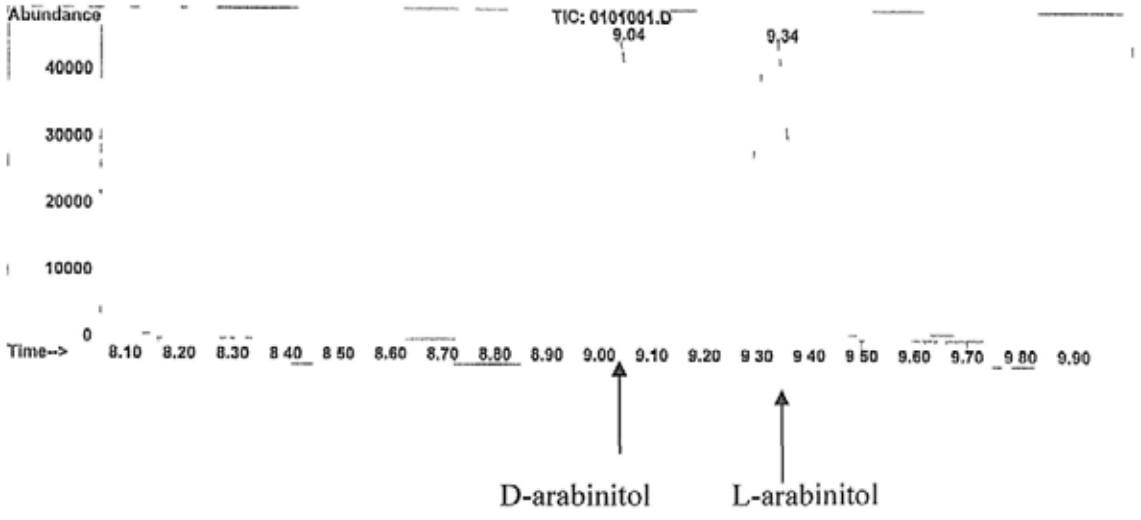


Table 8.1. D-/L- arabinitol ratios in various clinical groups.

Specimen type	Group	Nature of group	Number (n)	Mean	S.D.	Range	*P* value
Urine	A	Control –healthy adults	40	2.08	0.78	0.57 – 3.55	$P > 0.05$ (compared against groups: B, C, D,E)
Urine	B	Control - bacteriuria	40	1.94	0.56	0.76 – 3.18	$P > 0.05$ (compared against groups: A, C, D,E)
Urine	C	Control – oral candidiasis	15	1.77	0.60	0.91 – 3.26	$P > 0.05$ (compared against groups: A, B, D,E)
Urine	D	Control – vaginal candidiasis	15	1.87	0.52	0.94 – 3.11	$P > 0.05$ (compared against groups: A, B, C, E)
Urine	E	Control - candiduria	10	2.16	0.68	1.99 – 3.24	$P > 0.05$ (compared against groups: A, B, C, D)
Serum	F	Control – healthy adults	20	1.79	0.75	0.74 – 3.54	$P > 0.05$ (compared against group G)
Serum	G	Control - bacteremia	20	2.00	0.67	0.91 – 3.17	$P > 0.05$ (compared against group F)
Urine	H	Culture-proven candidemic group	7	9.91	3.04	7.24 – 16.27	$P < 0.01$ (compared against groups: A, B, C, D, E)
Serum	I	Culture-proven candidemic group	7	13.58	7.31	5.57 – 25.88	$P < 0.01$ (compared against groups: F, G)

Table 8.2. D-/L- arabinitol in Candidaemic patients' group.

Patient number	Diagnosis[a]	Serum D-/L-arabinitol ratio	urine D-/L-arabinitol ratio	Prior antibiotics use (days)	Presence of central venous lines	Causative candida species[b]	Outcome
1	AML	9.68	8.44	14	Yes	CALB	died
2	AML	5.57	7.24	16	Yes	CALB	died
3	50% Burns	10.5	9.08	10	Yes	CALB	died
4	AML	16.88	8.01	12	Yes	CTRO	died
5	AML	7.39	10.99	17	Yes	CKRU	died
6	SLE	19.16	16.27	15	No	CALB	died
7	IVDA with Candida endocarditis	25.88	9.34	8	No	CAPA	survived

[a] AML, acute myeloid leukaemia; SLE, systemic lupus erythematosus; IVDA, intravenous drug addiction.
[b] CALB, *C. albicans;* CTRO; *C. tropicalis*; CKRU, *C. krusei*; CAPA, *C. parapsilosis.*

Chapter 9:
Susceptibility Profiles

9.1 Background and Objectives

The importance of antimicrobial susceptibility testing is beyond doubt with clinical bacteriology. Emergence of resistance is a constant threat as bacteria evolved rapidly to combat against antimicrobials. The situation with fungus is less clear. Widespread use of fluconazole, after its introduction in the 1990s, has recently raised a concern on emerging antifungal resistance. However, routine susceptibility testing was technically difficult due to the lack of standardization, as well as interpretation criteria. Numerous methods such as macrobroth dilution test, microbroth dilution test and E-test had all been tried, but none has gained universal acceptance. In recent years, disk diffusion testing was established but is limited to fluconazole and voriconazole.

The problem was partially solved, when the commonest infecting fungus, *Candida*, was known to have a correlation between species and susceptibility results. *C. krusei*, for example, is intrinsically resistant to azole agent. Thus a confirmed speciation can help to guide antifungal usage. On the other hand, *C. glabrata*, exhibited susceptible dose-dependent (S-DD) phenomenon, in which a high dose (and could be more toxic) of azole drug is needed. Taking these together, while routine susceptibility testing of individual *Candida* may not be needed, a surveillance

programme should be instituted.

Apart from antifungals, other 'chemical' had been traditionally used. For example, gentian-violet paint and lavender oil for superficial / vaginal candida infection (D'Auria 2005; Watson 2007). Boric acid is used in some countries as vaginal pessaries in the treatment of vaginal thrush, and may offer a possible route for superficial decolonization (van Slyke 1981).

It is with this in mind, that the following studies were done to explore the susceptibility profile of the *Candida* species.

9.2 Study eight: In vitro activity of fluconazole and voriconazole against invasive *Candida* isolates in patients with candidaemia from 2001 – 2008.

9.2.1 Abstract

Antifungal treatment ranges from conventional amphotericin B to the new echinocandins. Azole agents provide an alternative choice because oral formulation is available. Fluconazole and voriconazole has been used in the treatment of candidaemia for several years, thus, it is important to monitor for the trend of antifungal resistance. Non-duplicate *Candida* isolates collected from candidaemic patients from 2001 to 2008 were tested against fluconazole and voriconazole in accordance to the NCCLS (CLSI) standard M44-A by disk diffusion method. A total of 143 *C. albicans,* 37 *C. parasilopsis*, 38 *C. glabrata*, and 57 *C. tropicalis* isolates were available for testing. Among them, only 6 *C. albicans* (4.2%) revealed non-susceptibility to fluconazole and/or voriconazole. *C. parapsilosis*, *C. tropicalis* and *C. glabrata* showed resistance to fluconazole at 0%, 14% and 39.5% respectively. That of voriconazole was 0%, 7% and 2.6 % respectively. Thus, even though fluconazole has been under extensive use for many years, its resistance rate against *C. albicans* and *C. parapsilosis* remains very low. Voriconazole has slightly better effect than fluconazole. Twenty-two patients (8%) had prior exposure to azole agents. In terms of development of *C. glabrata* or azole non-susceptible isolates, no significant difference was observed between those who had or had not received prior azole treatment.

9.2.2 Methods

9.2.2.1 Clinical isolates

From 2001 to 2008, non-duplicate blood culture isolates representing invasive infections were stored at -70°C by the dept of microbiology, of the Chinese University of Hong Kong. The laboratory utilized an automated continuous monitoring blood culture system (BacT/Alert system, Organon Teknika Corp., Durham, N.C.). Organisms were identified with standard microbiological methods using Gram's stain, germ tube test, CHROMagar appearance, Cornmeal appearance, and carbohydrate utilization tests (API 20C AUX or 32C AUX, bioMerieux, Hazelwood, Mo.; Vitek 2, bioMérieux, Marcy l'Etoile, France).

9.2.2.2 Disk diffusion testing

The disk diffusion was done according to CLSI (formerly NCCLS) M44-A disk diffusion method. *Candida* organisms adjusted to 0.5 McFarland standard were lawned onto methylene-blue-glucose agar (Mueller-Hinton agar supplemented with 2% glucose and 0.5 μg of methylene blue per ml). Fluconazole (25-μg) and voriconazole (1-μg) disks (Becton Dickinson, Sparks, Md.) were tested. All plates were incubated in air at 35°C and read at 24 hr. Zone diameter were read at 80% growth inhibition by using an image analysis plate reader system (BIOMIC, Giles Scientific, Calif.).

Control strains used were *C. albicans* ATCC 90029 and *C. parapsilosis* ATCC 22019. The interpretive criteria for the fluconazole and voriconazole disk diffusion tests were: susceptible, zone diameters of ≥19 mm (fluconazole) and ≥17 mm (voriconazole); susceptible dose dependent (SDD), zone diameters of 15 to 18 mm (fluconazole) and 14 to 16 mm (voriconazole); and resistant, zone diameters of ≤14 mm (fluconazole) and ≤13 mm (voriconazole).

9.2.2.3 Prior usage of azole agents.

Patient's chart review was conducted to identify prior systemic usage of azole agents (fluconazole, voriconazole) within one year (prior to the onset of candidaemia) or during the same hospitalization (whichever was longer).

9.2.2.4 Statistical analysis

Categoric variables were analysed by Chi-square test or Fisher's exact test where appropriate.

9.2.3 Results

From 2001-2008, a total of 275 isolates were tested, namely 143 *C. albicans* (55.6%), 37 *C. parapsilosis* (13.5%), 38 *C. glabrata* (13.8%) and 57 *C. tropicalis* (20.7%) were tested.

For *C. albicans*, only 6 (4.2%) isolates demonstrated non-susceptibility to fluconazole and/or voriconazole, one of them showed cross resistance to both drugs. For *C. parapsilopsis*, no resistance was observed against fluconazole or voriconazole. For C. *tropicalis*, fluconazole resistance was observed in 8 (14%) isolates, 4 (7%) of them were also resistant to voriconazole. For *C. glabrata*, fluconazole resistance was observed in 3 (7.9%) isolate and SDD in 12 (32%). Only 1 (2.6%) isolate showed cross resistance to voriconazole (table 9.1).

29 patients with fluconazole or voriconazole non-susceptibility were identified, prior usage of azole agents was present in 2 of them. Of the remaining patients with azole susceptible *Candida* isolates, 20 of them had prior usage of azole agent. No statistical significant difference was observed between the groups ($p > 0.05$).

Of the 38 patients with *C. glabrata* infections, prior usage of azole agents was found in 3 of them. For the 237 patients with non-*C. glabrata* infections, 19 patients had prior azole exposure, no statistical significance was observed between these two groups ($p > 0.05$).

9.2.4 Discussion

Among the antifungal agents, orally available agents are uniquely placed, as they

are convenient for prophylaxis purpose, and allow easier administration for patients who require prolonged therapy after the acute infective episodes, or for deep fungal infections. The use of fluconazole prophylaxis in HIV/AIDS in the primary or secondary prevention of cryptococcal infections had remarkably reduced their morbidity and mortality (Chang 2005). It is also used in patients after solid organ transplant (Hadley 2009; Lortholary 1997). Voriconazole and fluconazole have been widely used in heamatological malignant patients for prophylaxis prior to chemotherapy or bone marrow transplantation (Marr 2008). However, resistance had emerged (Millon 1994; Sangeorzan 1994). While there was no obvious rise in azole resistance rates, continuous surveillance would be warranted.

Our results were consistent with previous studies (Pfaller 2005, 2007). Large proportion of *C. glabrata* demonstrated non-susceptibility. *C. albicans* and *C. parasilopsis* were persistently susceptible to azole agents despite widespread usage. Pfaller *et al* reported in 2007 on an 8.5 year global surveillance and found that fluconazole was active against 97.9% of *C. albicans* and 93.3% of *C. parapsilosis*. In his series, only 68.9% of *C. glabrata* was susceptible to fluconazole. Our *C. tropicalis* appeared to be slightly more resistant at 14%. As the number of isolates was relatively small, a larger sample size would be able to clarify the picture. Overall,

voriconazole appeared more active against these fluconazole resistant isolates.

Prior exposures to azoles, especially long term fluconazole suppressive therapy used in HIV patients, were known risk factor for the development of azole resistance (Heinic 1993, Sanguineti 1993). In this study, 22 patients had prior utilization of azoles, and these did not affect the subsequent isolation of azole resistant isolates nor *C. glabrata*. None of them was HIV patients requiring prolonged azole prophylaxis. Furthermore, majority of these patients received their azole in in-patient settings, thus there was less chance for non-compliance. A larger sample size would be useful to define if different host factors will affect the development of azole resistance. None the less, prudent use of antifungal is important. One way to reduce azole exposure is probably through discontinuation of secondary prophylaxis in patients who are no longer immunocompromised (Nwokolo 2001; Sheng 2002).

The recent establishment of disk diffusion testing has enabled testing to be done in routine laboratories. Methylene-blue-glucose agar is easy to prepare but needs to be freshly made every week. This may not be adoptable for routine usage if the case number is small. The use of automated image system to capture the zone sizes also enables reading errors or biases to be removed. This will allow inter-laboratory

comparison of results (Doczi 2006). Base on this, it should be feasible for laboratories to test their *Candida* isolates on a monthly or quarterly basis, as part of the surveillance programme.

Table 9.1. Fluconazole and voriconazole susceptibility results against *Candida* species.

Isolates (*n*)	Fluconazole (%)			Voriconazole (%)		
	S	SDD	R	S	SDD	R
C. albicans (143)	138 (96.5)	2 (1.4)	3 (2.1)	140 (97.9)	1 (0.7)	2 (1.4)
C. parapsilosis (37)	37 (100)	0	0	37 (100)	0	0
C. tropicalis (57)	49 (86)	0	8 (14)	53 (93)	0	4 (7)
C. glabrata (38)	23 (60.5)	12 (31.6)	3 (7.9)	37 (97.4)	0	1 (2.6)

9.3 Study nine: In vitro susceptibility testing of vaginal *Candida albicans* isolates against boric acid and its possible antifungal mechanisms

9. 3.1 Abstract

Boric acid therapy for the treatment of vulvovaginal candidiasis was described more than 30 years ago. It is envisaged that this agent may be useful in treating patient heavily colonized with *Candida*. In this study, we aimed at evaluating the susceptibility profile of vaginal *C. albicans* isolates to boric acid and its antifungal mechanisms. A total of 12 vaginal yeast isolates of *C. albicans* were tested for boric acid MIC determination at 24hr and 48 hr. At 24 hour, all vaginal isolates and types strains of *Candida* demonstrated an MIC of >32mg/L against boric acid, with the exception of *C. krusei* ATCC 6258 which showed an MIC of 32mg/L. By 48 hours, the *C. krusei* ATCC 6258 also demonstrated an MIC of >32mg/L. At a concentration of 5000mg/L, boric acid inhibited biofilm formation and hyphal growth at 4 hr and 24 hr. Despite the high MIC values recorded, boric acid is administered topically at a concentration much higher than was used in our test system. It is thus plausible that the fungus be inhibited by the highly concentrated drug *in vivo*, resulting in apparent clinical cure.

9.3.2 Methods

9.3.2.1 Candida isolates

A total of 12 vaginal yeast isolates of *C. albicans* were tested. Type strains used include *C. albicans* ATCC 24433 and ATCC MYA-2876.

9.3.2.2 MIC determination

Boric acid (Sigma-Alderich, USA) in different concentrations was incorporated into Petri plates with RPMI 1640 medium with final concentrations ranging from 0.015-32 mg/L. The minimum inhibitory concentration (MIC) value was recorded as the lowest concentration that inhibited visible growth after 24h of incubation; and also again after 48h of incubation

9.3.2.3 Effect of boric acid on biofilm formation

Candida biofilm formation was studied in a static model. *C. albicans* (ATCC MYA-2876) were grown on Sabouraud agar plates for overnight. Fresh colonies were diluted with YNB to 10^7cfu/mL. Aliquots of 1mL cell suspension was placed in flat-bottomed polystyrene 6-well cell culture plates (Corning, N.Y.) and allowed to incubate at 37°C for 1 hr. Non-adherent cells was then washed off by gentle pipetting

and washing with YNB for thrice. Fresh YNB with or without boric acid at a concentration of 5000mg/L was refilled into the wells. Blank control contained YNB alone. These were then allowed to incubate at 37°C for 24 hr. Afterwards, the cell suspension and YNB was washed with distilled water once. Safranin was used to stain the wells and subsequently washed with distilled water once. One mL distilled water was replaced into each well. Biofilm formation was assessed spectrophotometrically with a plate reader at 540nm (Shin 2002). Tests were performed in triplicates and repeated twice.

9.3.2.4 Effect of boric acid on Candida albicans hyphal formation

C. albicans (ATCC MYA-2876) was grown on Sabouraud agar for overnight before use. A single colony was suspended into 10% horse serum with and without 5000mg/L boric acid. These were then incubated at 37°C for up to 24hr. Hyphal formation was inspected microscopically and recorded at time zero, 4hr and 24hr (Nion Eclipse TE 2000S) (de Seta 2009). Tests were performed in triplicates.

9.3.2.5 Statistical analysis

Continuous variables were analysed by Student's *t* test.

9.3.3 Results

At 24 hour, all vaginal isolates and types strains of *Candida* demonstrated an MIC of >32mg/L against boric acid, with the exception of *C. krusei* ATCC 6258 which showed an MIC of 32mg/L. By 48 hours, the *C. krusei* ATCC 6258 also demonstrated an MIC of >32mg/L.

Biofilm formation with or without treatment by boric acid was shown in figure 9.1. Treatment with 5000mg/L boric acid decreased the absorbance to nearly baseline level as observed with blank YNB ($p < 0.05$).

Hyphal (germ tube) formation in 10% horse serum without boric acid was clearly observable at 4 hr of incubation. By 24 hr, long filamentosus hyphae can be seen. In contrast, hyphae was absent in cells suspension with boric acid (figure 9.2).

9.3.4 Discussion

Boric acid is used as a preservative for decades in the microbiology laboratory. It bacteriostatic effect is useful for suppressing overgrowth of bacteria in urine sample (Meers 1990). Its use as a vaginal suppository to treat vaginal candidiasis have been common practice in some countries, but not in our locality (das Neves 2008). The usual dose is 600mg daily for 14 days (Ray 2007). Reports had suggested that boric acid can

be used as cure in azole resistatnt vaginal candidiasis (Shinohara 1997; Sobel 2003). While boric acid is renal toxic when taken orally, its topical use could be explored (Sabuncuoglu 2006). Recently, Aviñó-Martínez *et al* reported treatment of *Aspergillus* infected exenterated orbit with irrigation of boric acid (Aviñó-Martínez 2008). Thus, it might be a useful agent to for superficial *Candida* decolonization. However, before any clinical recommendation can be made, it is important to assess its basic fungicidal effect.

Our MIC data showed that basically all Candida species showed an MIC of 32mg/L or above. This should be interpreted with caution as standardized method of testing is yet to be available. Nevertheless, such high MIC suggests that boric acid will need to given in very high concentration for any observable antifungal effect. Recently, de Seta *et al* reported that the MIC for boric acid against *C. albicans* was 1563 to 6250 mg/L. Since the solubility of boric acid was 20,000 mg/L, so a 600mg dose will take 9mL to dissolve. It is predictable that in the vagina, the drug wont completely dissolve, thus attaining extremely high local concentration to exert antifungal effect (de Seta 2009).

Boric acid treated *C. albicans* had demonstrated inhibition of biofilm formation and hyphal formation at both 4 hr and 24 hr. Since hyphal formation was considered one of the virulence factor in *Candida* infection, this may explain the observed clinical usefulness of boric acid. This phenomenon was also similarly observed by de Seta *et al*,

who suggested that boric acid was fungistatic unless the culture time was prolonged. The inhibition of hyphal growth was likely an explanation for the fungistatic event. It would be of interest to explore this further with clinical isolates.

Boric acid cannot be administered systemically due to its toxicity. However, local usage may prove to be of value, especially in refractory cases which do not respond to usual antifungal treatment. Exploration of its antifungal mechanism would establish scientifically its value in the era of evidence based medicine.

Figure 9.1. Effect of boric acid on *C. albicans* biofilm formation.

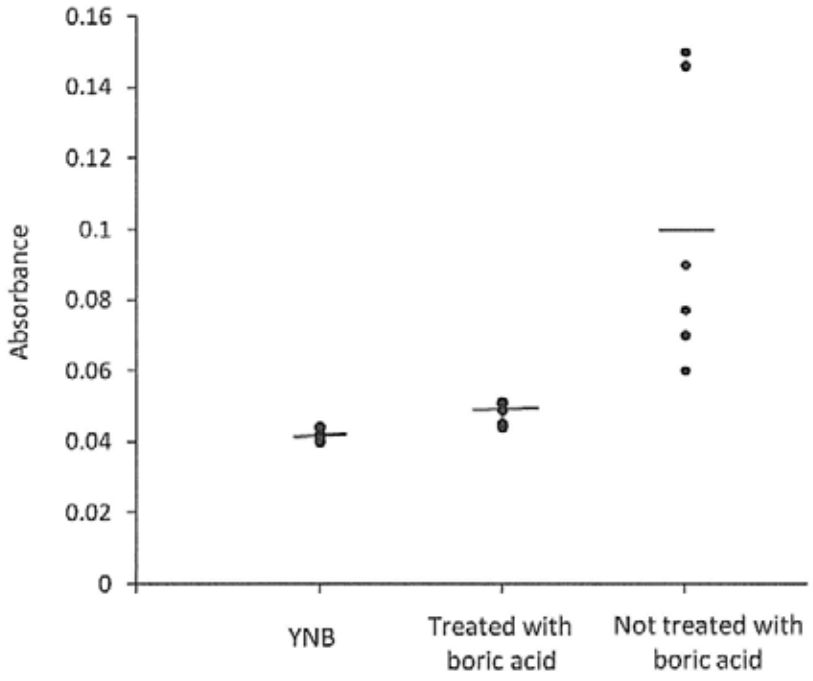
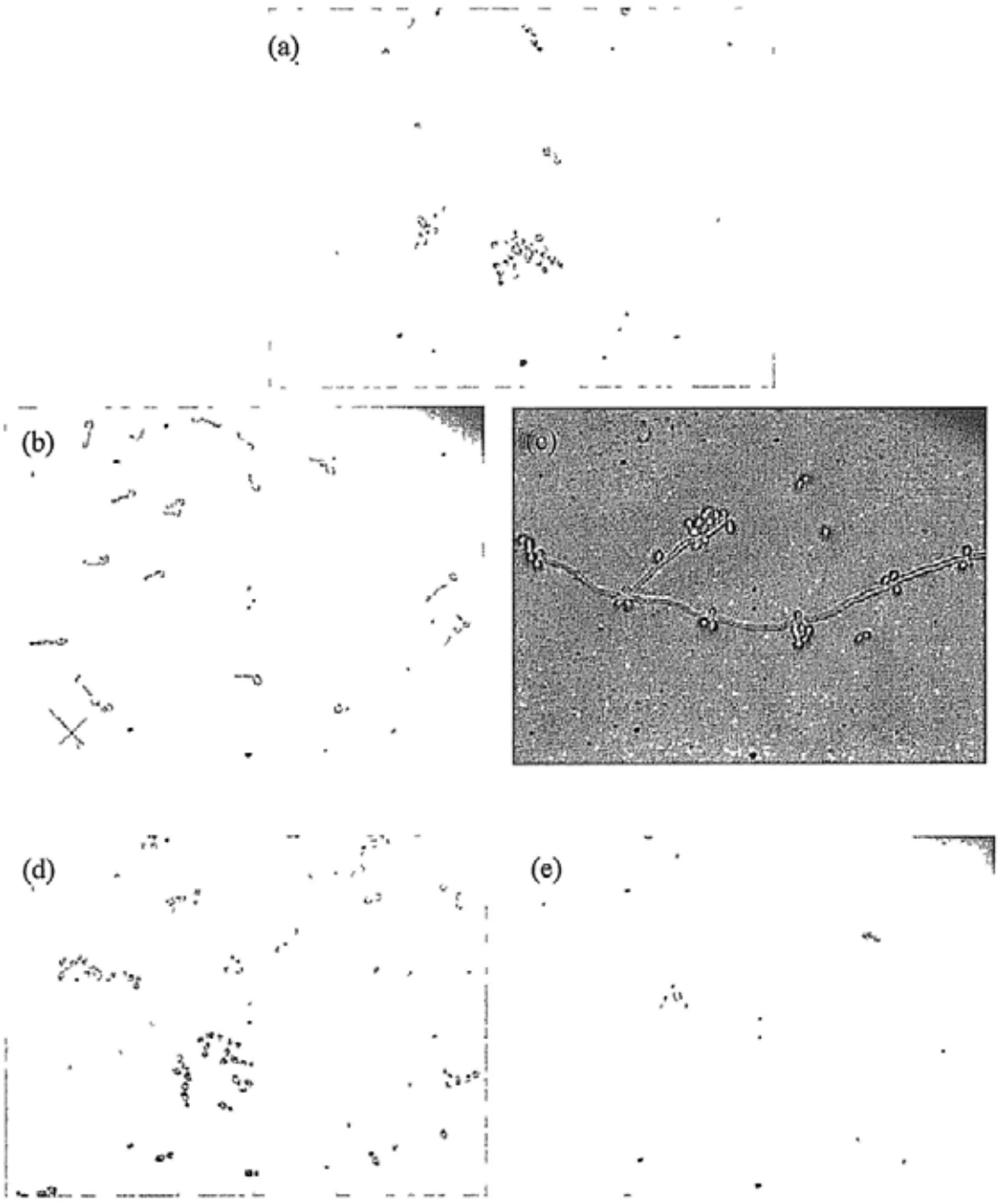


Figure 9.2. Effect of boric acid on hyphal formation of *C. albicans* observed at 400X magnification. (a) *C. albicans* at time zero; (b) C. albicans incubated for 4 hr without boric acid; (c) *C. albicans* incubated for 24 hr without boric acid; (d) *C. albicans* at 4 hr in the presence of boric acid at 5000mg/L; (e) *C. albicans* at 24 hr in the presence of boric acid at 5000mg/L.



Chapter 10 : Pathogenesis and the *ALS* genes

10.1 Study ten: Detection of *ALS1, ALS5* and *ALS6* genes in invasive Candida *albicans* isolates.

10.1.1 Abstract

ALS gene gamily encodes als proteins which are adhesins and contribute to biofilm formation. Several *ALS* genes (*ALS1*, *ALS5*, *ALS6*) were known to be adherent onto human fibroblast and vascular catheter materials. Cases of vascular catheter associated candidaemia by *C. albicans* were collected during 2005-2008 (positive growth from blood culture and vascular catheter culture). Eleven cases of catheter-related *C. albicans* bloodstream infections and 8 non-catheter related candidamic isolates were identified. These isolates were tested for the presence of *ALS1*, *ALS5*, and *ALS6* genes. Nine, 8 and 7 out of 11 catheter-related candidaemic isolates were positive for *ALS1, ALS5* and *ALS6* gene. Five of them were positive for all three genes. All 8 non-catheter related candidaemic isolates were negative for *ALS1* and *ALS6*. Three of them had *ALS5*. These results suggested that *ALS1* and *ALS6* were likely to play a role in catheter-associated candidaemia.

10.1.2 Background and objectives

Adhesion is an important process for infection to establish. The initial phase of organism attachment onto biological or artificial surface is mediated through adhesion proteins. In *Staphylococcus aureus*, adhesion is mediated through glycocalyx production. This subsequently developed into a biofilm, which harbors the sessile phase of the organisms, proctecting them from host immune response and antibiotic penetration, and releasing them periodically as persistent infection nidus (Clarke 2006; O'Gara 2007). In *C. albicans*, the biofilm formation is an important factor which allows the organisms to adhere onto bioprosthetic surface (Chandra 2001; Kojic 2004; Nobile 2006). In 1995, Hoyer *et al* described the agglutinin-like sequence (*ALS*) gene in *C. albicans* and postulated its potential role in adhesion (Hoyer 1995). Subsequently, nine *ALS* genes (*ALS1 – ALS9*) were described and they encode for a myriad of cell surface glycoproteins (Hoyer 2001a; Sheppard 2004). These Als proteins were found to mediate adherence onto various substrata, including gelatin, laminin, human umbilical vein endothelial cells, buccal epithelial cells, fibronectin and FaDu epithelial cells (Zhao 2004, 2005; Oh 2005). The *ALS* gene family has 8 members, namely *ALS* 1 to 9 (*ALS* 8 was now considered the same locus as *ALS3*) located in different chromosomes, and characterized by varying numbers of 108bp

tandem repeats, resulting in different lengths of the genes. This gene family has also been detected in *C. tropicalis*, and *C. dublinensis* (Hoyer 2001b), but their significance is mush less understood. It had been demonstrated through a *Saccharomyces cerevisiae* transformation model that *ALS1*, *ALS5* and *ALS6* confers adhesion to human fibroblast. In a catheter adhesion model *ALS1*, and *ALS6* had contributed to adhesion onto vascular catheter (Chan 2006). In this experiment, we explored the presence of these *ALS* genes in invasive *C. albicans* isolates.

10.1.3 Methods

Catheter-associated-candidaemia was defined as positive culture of *Candida* from both blood cultures and the removed intravenous catheter tip. Candidaemia with negative culture from catheter tips were considered as non-catheter-related. From the year 2005 to 2008, all cases of culture proven candidaemia were checked against the laboratory log for the simultaneous presence of vascular catheter culture. The microbiology laboratory used a semiquantitive method to culture vascular catheters. Briefly, a 5-cm segment of the catheter tip was cut aseptically upon removal. It was then cultured on blood agar and chocolate agar by the roll plate method. All culture will be incubated for 48hr before discard as negative. A colony count number of > 15

was considered as probable catheter related infection (Maki 1977). All microorganisms were identified according to standard laboratory procedures. The blood culture isolates of these cases were archived and stored in glycerol broth at -70°C before use.

A single colony of yeast cells was suspended in 200ml lysis buffer (100mM Tris-HCl, pH 7.5, sodium dodecyl sulphate solution 0.5% w/v, 30mM EDTA). The mixture was incubated at 100°C for 15 minutes, 100ml of 2.5M potassium acetate was then added and mixed. After being stored on ice for 60 min, it was centrifuged at 12000rpm for 5 min. The supernatant was transferred to a new tube. DNA was precipitated with an equal volume of isopropanol, washed with 0.5ml of 70% ethanol, air-dried and subsequently resuspended in 50ml of distilled water. 0.5ml of this DNA solution was utilized as templates in the PCR reactions. Primers were designed to flank the region of *ALS1*, *ALS5* and *ALS6* of *C. albicans* ATCC MYA-2876. The reaction mixture consisted of 20μL PCR supermix (Invotrogen, CA, USA), 1μL template (100ng/ul), 1μL of forward and reverse primers. Throughout the PCR procedures, precautions were taken to avoid contamination of the PCR products (Kwok 1989). Negative controls with reagent mixture were included in each run. *C. albicans* ATCC 90028, *Saccharomyces cerevisiae* ATCC 96686 were also included as negative control. *C. albicans* SC5314 (ATCC MYA-2876) were used as positive

control strain.

10.1.4 Results

Eleven blood culture isolates of *C. albicans* related to catheter infections and another 8 without catheter infections were selected for testing. Among the 11 catheter-related candidaemic isolates, 9, 8 and 7 isolates were positive for *ALS1*, *ALS5* and *ALS6* respectively. For the two *ALS1* negative strains, one was positive for *ALS5* and *ALS6* (strain no: 44381), the other was positive for *ALS5* only (strain no: 58999). Two strains were only positive for *ALS1* but not *ALS5* or *ALS6* (strain no: 62609, 53628). Another strain was positive for *ALS5* only (strain no: 58999). No strain was only positive for *ALS6* (table 10.1).

Among the eight non-catheter-related candidaemic isolates, none of them had *ALS1* nor *ALS6*. Three of them had *ALS5* (strains: 52974, 59928 and 75508).

10.1.5 Discussion

Discovery of *ALS* gene family was a recent advancement in mycological research. *ALS1* was first described by Hoyer in 1995 (Hoyer 1995). Although the whole gene

family had now been described, our understanding of it is far from complete. Apart from adhesion, recent research had discovered other functions of these genes. For example, it is now known that als3 proteins are structurally similar to clumping factor of *Staphylococcus aureus*. This could lead to misidentification of the organisms if slide coagualse test is performed without confirmation by tube coagulase test. On the other hand, there was also encouraging development such as cross kingdom vaccination to protect *Staphylococcus* infection (Spellberg 2008). Although the *ALS* genes are functionally diverse, the *ALS1* was considered to be a major contributor of adhesion, while the others play variable roles. In this study, we hope to explore the ALS genes and their relationship with clinical isolates.

In the catheter-related *Candida* isolates, one would expect to reveal *ALS* genes in the majority of the cases, especially *ALS1,* which was considered a critical component in the development of biofilm (Nobile 2008). This was true in our series, as 9 out of 11 isolates were positive for *ALS1* and 7 were positive of ALS6. In contrast, none of the non-catheter related ones were positive for these 2 genes. However, it was also known that the presence of *ALS* genes is not universal in *C. albicans* (Hoyer 2001a). Thus virulence via adhesion from a single gene family is unlikely the sole factor in the pathogenesis of catheter related candidaemia. In fact, it had been suggested that the

hwp1 proteins could also confer adhesion property in *C. albicans* (Padovan 2009; Soll 2008). The occurrence of *ALS5* did not differ significantly between the two groups. This suggested that the presence of *ALS5* may not correlate with catheter-related candidaemia. Moreover, *ALS5* can be naturally deleted and result in clade difference (Zhao 2005). A larger cohort of isolates will allow this to be examined more closely.

Five of the isolates from catheter related infections were positive for all three genes, while none occurred in the non-catheter related group. On the one hand it seems that the presence of *ALS* genes is an attractive explanation for catheter related candidaemia; on the other hand, the mere presence of the adhesion encoding gene does not translate into functionality directly. The expression of these genes, their regulations, and their inter-relationship with other genes, would shed further information as to how *Candida* adhesion is regulated (Cheng 2005; O'Connor 2005). Nailis *et al* demonstrated that the *ALS1-5* were upregulated during adhesion in both *in vivo* and *in vitro* models (Nailis 2010). Similarly, Green *et al* had also observed the expression of *ALS1-4* from two *C. albicans* strains in a rat hyposalivary model (Green 2006). Formation of the fungal biofilm is a complex process involving not only the *ALS* genes, but also other adhesins and filamentation to result in a mature biofilm. *EFG1* was shown to be involved in regulation of hyphal formation (Garcia-Sanchez

2004; Sohn 2003); *EAP1* was associated with the expression of cell surface adhesion (Li 2003); *RBT1*, *RBT5*, and *WAP1* also encoded cell wall proteins (Braun 2000).

Our collection of strains was unfortunately quite small in number, thus a generalization of the results would not be possible. A larger collection of strains, simultaneous testing of the blood culture and catheter-tip isolates, and assessment of various gene expression by real-time PCR will generate more comprehensive results for analysis.

In summary, the results suggested that the majority of catheter related candidaemic isolates showed the presence of *ALS1* and *ALS6* genes. These genes encode adhesins that could contribute to the virulence of the organism. Future research should address the expression of these genes in various clinically relevant settings.

Table 10.1. Distribution of *ALS1*, *ALS5*, and *ALS* 6 in *C. albicans*.

Strain no:	Year of isolation	Catheter-related candidaemia (Yes/No)	*ALS gene*		
			ALS1	*ALS5*	*ALS6*
			(Presence: +; Absence: -)		
44381	2006	Yes	-	+	+
44669	2007	Yes	+	+	+
51776	2008	Yes	+	+	+
48466	2008	Yes	+	+	+
53628	2006	Yes	+	-	-
54986	2008	Yes	+	+	+
55311	2005	Yes	+	+	+
58999	2007	Yes	-	+	-
62609	2006	Yes	+	-	-
63111	2005	Yes	+	-	+
65219	2006	Yes	+	+	-
82136	2008	No	-	-	-
60864	2008	No	-	-	-
52974	2008	No	-	+	-
65822	2008	No	-	-	-
59928	2007	No	-	+	-
81074	2008	No	-	-	-
69676	2007	No	-	-	-
75508	2007	No	-	+	-

PART III : CONCLUSIONS

Chapter 11:
Results Summary and Discussions

This series of studies conducted over the past years was an attempt to understand infections caused by *Candida*, *Pneumocystis* and *Cryptococcus*. It was evident that a number of changes had occurred.

A definite improvement in the awareness of the disease among healthcare workers was observed. Instead of disregarding fungi as mere contaminant, clinicians are now more alert to the impact of invasive fungal infections. This is most probably due to the expanding knowledge on these organisms.

Cryptococcus and *Pneumocystis* infections affect immunocompromised and HIV/AIDS patients. In our locality with low HIV prevalence, it was observed that the many of these infections were unrelated to HIV co-infection. In fact, patients with varying degree of immunocompromisation due to other causes were affected. This resulted in an interesting phenomenon: these infections were not confined to the realm of infectious diseases specialty. Rather, these infections could present themselves to any medical specialty, even surgical specialty (such as the case of hydrocephalus complicating *Cryptococcus* infections). Therefore an understanding and awareness of these infectious should be borne by any medical practitioner. This could be achieved through post-graduate or even undergraduate training programmes.

In candidaemia, the decline in *C. albicans* infections was consistent with findings from other parts of the world. Studies had shown that this decline had

started to plateau. In clinical practice, it would be reasonable to expect half of the candidaemic cases are due to *Candida* non-albicans. Since there is a direct relationship between *Candida* species and antifungal susceptibilities, it is desirable to speciate all invasive *Candida* organisms. An understanding of the intrinsic resistance in various *Candida* species would help clinicians to choose the most appropriate therapy.

A number of new antifungals were developed in the past 10 years. Among them, the echinocandins represents a major breakthrough. They are selectively toxic when compared to azoles and polyenes. If the species of *Candida* are unknown, the echinocandins are the drug of choice in empirical treatment.

Standardized antifungal susceptibility testing is now possible. Although the rate of resistance is still low, it would be prudent to continue surveillance.

The mortality of our patients, unfortunately, remained high. As fungi are opportunistic pathogens, the underlying diseases and/or immunosuppressions are critical to the development of infections. If the risk factors are not modifiable, then the use of prophylactic antifungal therapy may be warranted. Green *et al* had advocated the use of prophylaxis for *Pneumocystis* infections if its risk is greater than 3.5% (Green 2007). Future mycological research should address this important clinical issue on prophylaxis.

There are now renewed interests in alternative antifungal therapy. Investigator had just started to investigate the antifungal mechanism of boric acid. Other remedies (especially herbal ones), such as lavender oil, would probably receive more attention in further research.

Outbreaks of infectious diseases have received unparallel attention. The public expect a high standard of medical care. All health care workers should strive for the best clinical skills, which also include infection control practices. Hospital acquired infections need to be detected promptly so that intervention can be done. Molecular typing methods are the most appropriate tool in this aspect. The infrastructure for molecular services is available in many clinical laboratories already, it would be appropriate to adopt typing techniques in the near future.

The research of pathogenic mechanism is now a rapidly evolving field. With the completion of genome projects on *Candida albicans* and other yeasts, it is now possible to search for virulence factors and drugs targets. We would expect a rapid expansion of our understanding in pathogenesis in the foreseeable future.

There are many other unanswered questions in clinical mycology. Antifungal combination therapy has always been a controversial issue. *In vitro* data is conflicting and *in vivo* data is scarce. Proper randomized controlled trials would be needed to address this issue. However, as most of these drugs are still under patency

and belongs to various pharmaceutical companies, getting them together for a proper clinical trial may not be accomplished in the near future.

Drug cost is a major concern, while amphotericin B deoxycholate costs US$25 per day at 50mg daily dose, liposomal amphotericin B (LAMB) cost US$650 per day at 150mg daily dose. This is prohibitory expensive for most patients. An emerging new specialty on "*pharmacoeconomics*" may be able to tell whether the much higher cost is indeed value for money.

While invasive yeast infections can be deadly, there are still much to look forward to. With further understanding of these diseases, we would expect continue improvement in the quality of our medical care.

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