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Genetic relatedness of *Candida albicans* bloodstream infection clinical isolates in Malaysia

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ABSTRACT

Aims: The aim of this study was to investigate the genetic relatedness of the most prevalent *Candida* bloodstream infection (BSI) species in in a Malaysian population via Randomly Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) fingerprinting.

Methodology and results: The genomic DNA of 43 *Candida* BSI blood culture samples obtained from Universiti Malaya Medical Centre (UMMC) was isolated, after which species identification was carried out using PCR with *ITS-1* and *ITS-4* pan-fungal primers in conjunction with CHROMagar™ *Candida*. The predominant *Candida* species in the BSI samples is *Candida albicans* (14 out of 43 isolates). RAPD-PCR on these 14 *C. albicans* clinical isolates was performed using *PST* as the arbitrary primer. Data analysis using MEGA found an overall non-relatedness of these 14 clinical isolates [average similarity coefficient (S_{AB}) value 0.733±0.172]. Following in-depth analysis, five of the 14 isolates were observed to be identical (S_{AB} values of 1.00 each), four isolates had S_{AB} values of 0.80-0.99, indicating that they are highly similar, but are non-identical, while five isolates are unrelated (S_{AB} lower than 0.80). This suggests that microevolution might have occurred and that these clinical isolates may possibly belong to different strains.

Conclusion, significance and impact of study: A fair degree of genetic heterogeneity was found among the 14 *C. albicans* isolates from UMMC. To our knowledge, this is the first report on the genetic profiles of *C. albicans* bloodstream infection isolates from Malaysia, warranting further studies in the possible evolutionary trends within this *Candida* species in Malaysia.

Keywords: Randomly Amplified Polymorphic DNA PCR (RAPD-PCR), *Candida albicans*, *Candida* bloodstream infections, Genetic relatedness, DNA fingerprinting

INTRODUCTION

In the last decade, the emergence of nosocomial bloodstream fungal infections in persons with challenged immune systems (such as those with extensive burns, HIV infections, leukopenia, organ transplantation and patients undergoing chemotherapy) has heightened the need for more extensive research, especially in the genetics aspect of the causative pathogen. One such fungal infection is *Candida* bloodstream infections (BSIs), which is caused predominantly by *Candida albicans* (Nguyen *et al.*, 1996; Pfaller *et al.*, 1998; Trtkova and Raclavsky, 2006; Moretti *et al.*, 2013). The primary origin of *Candida* BSIs has been the subject of debate (endogenous- or exogenous-acquired). Endogenous acquisition of bloodstream infections, from existing colonization of *Candida* from one's own gastrointestinal flora is the major source of *Candida* BSIs (Nucci and Anaissi, 2001; Magill *et al.*, 2006; Miranda et *al.*, 2009). The exogenous origin of *Candida* BSI is implied when the causative pathogen is acquired nosocomially. The transmission may occur through direct contact or indirectly via hospital personnel (hand carriage of *Candida* strains of healthcare worker, intravascular catheters and parenteral hyperalimentation) (Hedderwick *et al.*, 2000; Hota, 2004).

Candida BSIs occur when *Candida* spp. opportunistically penetrates the bloodstream through breaks or cuts in the skin or mucinous membranes. When *Candida* cells enter the bloodstream, they easily spread

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throughout the body and infect the various organs in the body. As a consequence, invasive *Candida* adapt rapidly to the new environment by expressing a distinct set of genes. These genes and the products contribute to fungal pathogenicity and are described as virulence factors (Odds, 1994).

With high crude mortality rates of 35-67% (Pelz *et al.*, 2000; Marriott *et al.*, 2009; Moran *et al.*, 2010), reported incidences of *Candida* BSIs and the rank order of causative species differs significantly between countries. *Candida albicans* is noted to be the most common causative pathogen in countries like the United States, Europe, Australia and some Asian countries (Sandven 2000; Chong *et al.*, 2003; Chen *et al.*, 2006; Tay *et al.*, 2009; Cisterna *et al.*, 2011). Previous studies by Ng *et al*. (2006) demonstrated that *C. parapsilosis* and *C. tropicalis* surpassed *C. albicans* in terms of prevalence in Malaysia. However, the findings of Ng *et al.* (2006) contradicts studies performed by Tzar *et al.* (2009) and Amran *et al.* (2011), where *C. albicans* is identified as the predominant species isolated from *Candida* BSI patients in a Malaysian hospital setting. The slight discrepancy is apparent, as there is a dearth of reports on *Candida* spp. distribution in Malaysia and their biotypes as well as their genetic relatedness (Chong *et al.*, 2003; Ng *et al.*, 2006; Amran *et al.*, 2011).

Despite the prominence of nosocomial *C. albicans* as a major fungal pathogen, little is known about their genetic make-up, evolution and persistance during commensalism (Pires-Goncalves *et al.*, 2007). Over the past decades, advances in molecular biology have led to the delineation of *C. albicans* subtypes and has facilitated epidemiological recording and analysis (Pires-Goncalves *et al.*, 2007). RAPD-PCR fingerprinting techniques have proved to be useful for hospital epidemiology; in particular, for investigating infection clusters of invasive *Candida* BSIs, differentiating strains, investigating intra- and interspecies similarity and discriminating microevolution in a single colonizing strain (Soll, 2000; Pires-Goncalves *et al.*, 2007). As to date, very little studies pertaining to the genetic profiles of *Candida* BSIs at the intraspecies level have been performed in Malaysia, as most studies are designed for interspecies level (Chong e*t al.*, 2003). Hence, by employing the RAPD-PCR technique, the genetic relatedness of all the *C. albicans* clinical isolates in this study were assesed in order to observe their strain diversity at intraspecies level that may possibly contribute to different degrees of virulence, pathogenecity and treatment responses.

MATERIALS AND METHOD

Clinical Isolates

A total of 43 blood culture (BC) samples of *Candida* bloodstream infections (BSIs) sourced from Universiti Malaya Medical Centre (UMMC) were used in this study. All *Candida* BSI clinical isolates which were previously confirmed using the BD BACTEC™ 9240 system, were cultured onto the Sabouraud's Dextrose Agar (SDA) (BD, Difco) with incubation at 37 °C for 48 h.

Identification of *Candida* spp. via CHROMagar™ *Candida*

Single colonies of *Candida* spp. clinical isolates from SDA were streaked onto CHROMagar™ *Candida* (BD, Difco) and incubated at 37 °C for 48 h to observe the development of colony colors.

Identification of *Candida* spp. via Internal Transcribed Spacer (ITS)-1 and ITS-2 region amplification

Genomic DNA (gDNA) was isolated from each *Candida* clinical isolate according to the manufacturer's protocol (Analytik Jena, Germany). DNA amplification of the highly variable non-coding regions (ITS-1 and ITS-2) of the fungal ribosomal DNA (rDNA) was performed in volumes of 25 µL. Each reaction contained 2.5 µL of 10× *Taq* Buffer (supplemented with 1.5 mM $MgCl_2$), 0.5 µL of 10 mM dNTP, 0.5 µL of 1 U of *Taq* Polymerase (all from NEB, USA), 0.5 µL each of 10 µM *ITS1* (5'- TCCG TAG GTG AAC CTG CGG -3') and *ITS4* (5'-TCCT CCGC TTA TTG ATA TGC -3') primers, 1 µL of 40 ng DNA template and 19.5 µL of sterilized Milli-Q water. PCR was performed in a thermal cycler (Eppendorf Personal, Germany) with an initial denaturation of 95 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 sec, after which a final extension was performed at 72 °C for 5 min. The amplicons were then analyzed using 1.5% agarose gel electrophoresis.

RAPD-PCR assay

All clinical isolates identified as *C. albicans* were subjected to RAPD-PCR fingerprinting assay. RAPD-PCR was performed using *PST* as an arbitrary primer. Forty nanograms of each total DNA was added to 25 µL RAPD-PCR reactions, each containing 2.5 µL of 10× *Taq* Buffer (supplemented with 1.5 mM $MgCl_2$) 0.5 µL of 10 mM dNTP, 0.5 µL of 1 U of *Taq* Polymerase, 3 mM of $MgCl_2$ (all from NEB, USA), and 2 µL of 10 µM *PST* arbitrary-primer (5'-CAGTTCTGCAG-3').The PCR cycling condition was: initial denaturation at 95 °C for 5 min, followed by 45 cycles of denaturation at 94 °C for 30 sec, annealing at 49 °C for 30 sec and extension at 72 °C for 30 sec, and a final round of extension at 72 °C for 5 min (Eppendorf Personal, Germany). RAPD-PCR fingerprinting was performed in triplicates for each *C. albicans* clinical isolate as ascribed by Lamboy *et al.* (1994). The resulting amplicons were analyzed using 1.5% agarose gel electrophoresis.

Phylogenetic analysis

The RAPDistance program, version 1.04 (Armstrong *et al.*, 1994) was used to assess the relatedness of the 14 *C. albicans* clinical isolates. In the RAPDistance program,

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the presence of bands was scored with 1.00, while the absence of bands was scored with 0.00. The Dice metric coefficient (Dice, 1945) method was the statistical method chosen to calculate the similarity coefficient (S_{AB}) of each sample pair. S_{AB} was calculated according to Chong *et al*. (2003) as follows: $S_{AB} = 2n_{11} / (2n_{11} + n_{01} + n_{10})$, where n_{11} represents the number of bands shared by samples A and B, n_{01} represents the number of bands present in sample A but not in sample B and n_{10} represents the number of bands present in sample B but absent in sample A. Construction of the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) phylogenetic tree was based on the pairwise-distance (1- S_{AB}) matrix of the 14 *C. albicans* clinical isolates and was generated by the MEGA software package, version 5.05 (Tamura *et al.*, 2011).

RESULTS

Identification of *Candida* spp. via CHROMagar™ *Candida* and amplification of ITS-1-ITS-2 regions

Consolidation of the results of CHROMagar™ *Candida* and amplification of ITS-1 and ITS-2 regions of the 43 clinical isolates from Malaysian *Candida* BSIs showed *C. albicans* to be the predominant species in this cohort (n=14; 32.5%), followed by other non-*albicans Candida* species (n=29), comprising of *C. parapsilosis* (n=12), *C. glabrata* (n=8), *C. tropicalis* (n=8), *C. orthopsilosis* (n=1) and *C. krusei* (n=1) (Figure 1).

RAPD-PCR assay and UPGMA phylogenetic tree analysis for *Candida albicans*

As the predominant species, *C. albicans* clinical isolates were subjected to RAPD-PCR fingerprinting to investigate intraspecies variation within this cohort. From the RAPD-PCR profiles (Figure 2), eight distinctive polymorphic bands, ranging from 400 bp to 1.2 kbp, were generated. In the UMPGA phylogenetic tree in Figure 2, the 14 *C. albicans* clinical isolates were grouped into two main clusters, denoted as (a) and (b). The first main cluster (a) gave rise to three distinct sub-clusters, where the first sub-cluster consists of BC15, BC16, BC21, BC34, BC36, and BC37. BC28 is a sister taxon (a closely related strain) to the first sub-cluster. In addition, BC9 and BC31 were observed to cluster together in the second sub-cluster, while BC11 and BC33 were in the third sub-cluster. The second cluster (b) comprises of only one sub-cluster, which consists of BC39 and BC12, while BC3 was noted to be a sister-taxon to BC39 and BC12, respectively.

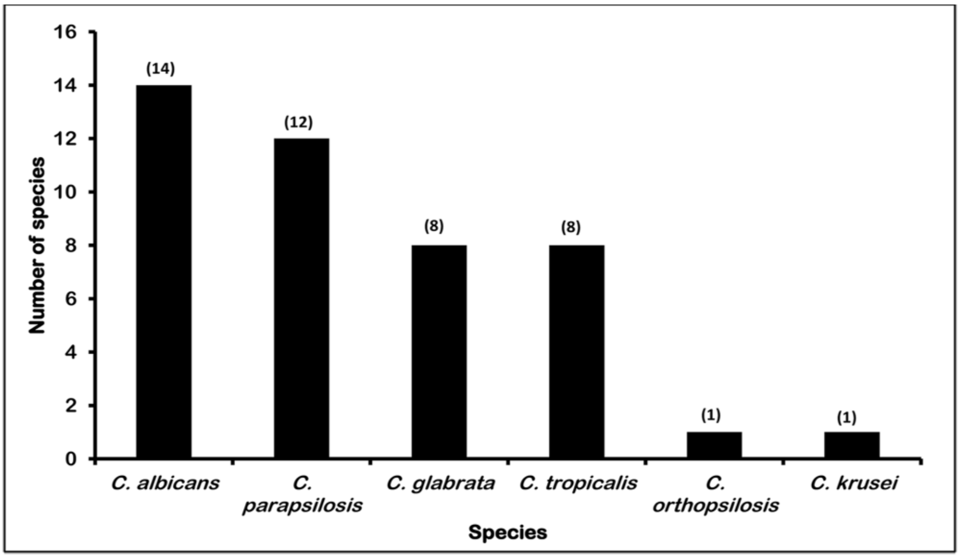

Figure 1: Species distribution of *Candida* spp. isolated from 43 Malaysian *Candida* BSIs. *C. albicans* is the most common species in this cohort.

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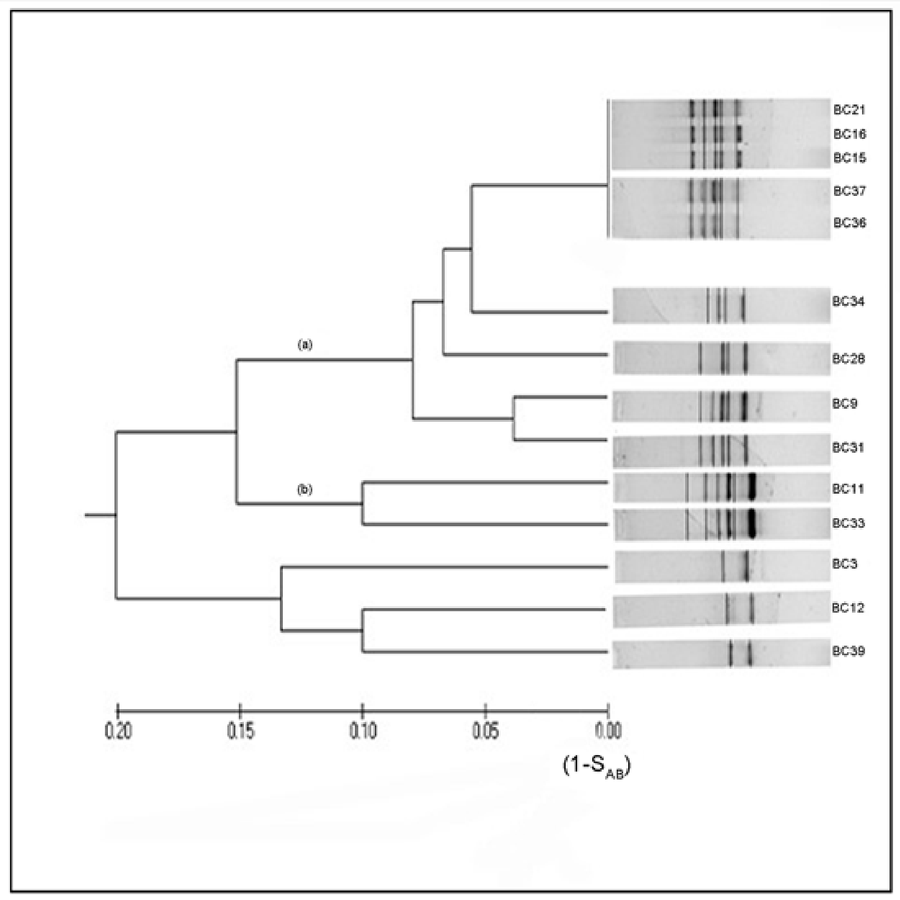

Figure 2: UPGMA phylogenetic tree generated from the similarity coefficients (S_{AB}). The S_{AB} values were determined by the Dice metric method from the RAPD-PCR patterns of 14 *C. albicans* BSI clinical isolates. The length of the horizontal lines in each cluster is proportional to the genetic distance ($1-S_{AB}$) and is drawn to the scale shown beneath the phylogenetic tree. BC denotes blood cultures. The phylogenetic tree was generated using MEGA 5.05 (Tamura *et al.*, 2011).

DISCUSSION

The results of this study indicate *C. albicans* (n=14; 32.5%) as the most common species from a total of 43 *Candida* spp. BSI clinical isolates in this Malaysian cohort (Figure 1). This study corroborates the findings of previous studies, where *C. albicans* was the most frequently isolated *Candida* spp. in *Candida* BSIs not only in Malaysia (Tzar *et al.*, 2009; Amran *et al.*, 2011), but also globally (Fridkin and Jarvis, 1996; Pfaller, 1996; Abi-said, 1999; Lim *et al.*, 2012).

For these 14 *C. albicans* clinical isolates, the RAPD-PCR method of analyzing intraspecies genetic relatedness using *PST* as an arbitrary primer resulted in distinctive and reproducible DNA fingerprints. No significant differences between biological triplicates of RAPD-PCR profiles were observed. Additionally, RAPD-PCR was also performed using different sets of arbitrary primers (*NS-02*, *OPE-04*, *OPE-08* and *OPA-02*). However, these primers were not suitable for further analysis because they yielded inconsistent banding patterns with a low discriminatory power despite several rounds of amplification.

Intriguingly, in the phylogenetic tree, most of the clinical isolates (9/14) could be placed into the major cluster (a), with a similarity index (S_{AB}) ≥0.80 (Figure 2). Of these, five *C. albicans* clinical isolates (BC15, BC16, BC21, BC34, BC36, and BC37) showed that they were genotypically identical (S_{AB} =1.00). Four other isolates (BC9, BC28, and BC31 and BC34) were considered highly similar but non-identical (0.80≤ S_{AB} ≤0.99). As for BC3, BC11, BC12, BC33 and BC39, these isolates were unrelated (S_{AB} < 0.80). The mean S_{AB} value for the 14 *C. albicans* clinical isolates was 0.733±0.172, which suggests overall intraspecies unrelatedness.

RAPD-PCR fingerprinting of the 14 *C. albicans* clinical isolates revealed a striking degree of variation in their genomes, which could not be differentiated using common conventional (CHROMagar™ Candida) and molecular (amplification of ITS-1 and ITS-2 region) methods of species identification. The RAPD-PCR fingerprinting profiles suggest that strain diversity exists

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within these *C. albicans* clinical isolates, and that they may have undergone microevolution for reasons that are yet unknown (Chong *et al.*, 2003). To investigate the occurrence of microevolution, blood samples from the same patient must be collected again after a period of one year, where the genetic relatedness of the newly isolated *C. albicans* is assessed and compared to the existing data using similar parameters adopted by this study.

In addition, in the present study, there is also a possibility that this group of isolates, though classified as *C. albicans* based on the phenotypic properties (CHROMagar™ *Candida*) and through molecular identification, could represent either a novel species or a novel strain closely resembling *C. albicans* or a sub-species within a species. Subsequently, from the RAPD-PCR fingerprint patterns generated in Figure 2, it is suggested that four patients (hosts for BC15, BC16, BC21 and BC36) could be infected with an identical *C. albicans* strain, whether acquired nosocomially or from the same source. Similar results were demonstrated by Boccia *et al.* (2002) in an Italian hospital, where *C. albicans* strains isolated from five infants with *C. albicans* BSI from neonatal intensive care units were genotypically identical, thus suggesting that cross-infection might have occurred among these infants.

In summary, the results from the present study demonstrated *C. albicans* to be the predominant species in this cohort of 43 Malaysian *Candida* BSIs. RAPD-PCR fingerprinting using *PST* as an arbitrary primer and phylogenetic analysis portrayed the overall intraspecies unrelatedness of the 14 *C. albicans* isolates. To our knowledge, this is the first study to report intraspecies genetic variation in *C. albicans* clinical isolates from BSIs in Malaysia. The present findings have provided a glimpse into the population structure of Malaysian *C. albicans* BSI clinical isolates and are hoped to contribute to more robust epidemiology recordings of *Candida* spp. BSIs in Malaysia. Although the sample size of this study is relatively small, the data thus far warrants further studies on the genetic relatedness of both *C. albicans* and non-*albicans* BSI isolates to better understand the role of strain differences in virulence and pathogenicity.

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