# Antimicrobial susceptibilities and random amplified polymorphic DNA-PCR fingerprint characterization of *Candida glabrata*, *Candida parapsilosis* and *Candida rugosa* from two major hospitals in Kuala Lumpur, Malaysia

Madhavan, P.[1,2*], Chong, P.P.[3], Farida, J.[2], Fauziah, O.[4], Cheah, Y.K.[5], Arunkumar Karunanidhi, A.[2] and Ng, K.P.[6]

[1]School of Medicine, Faculty of Health and Medical Sciences, Taylor's University, No. 1, Jalan Taylor's, 47500 Subang Jaya, Selangor Darul Ehsan, Malaysia
[2]Department of Medical Microbiology and Parasitology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor Darul Ehsan, Malaysia
[3]School of Biosciences, Faculty of Health and Medical Sciences, Taylor's University, No. 1, Jalan Taylor's, 47500 Subang Jaya, Selangor Darul Ehsan, Malaysia
[4]Department of Human Anatomy, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor Darul Ehsan, Malaysia
[5]Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor Darul Ehsan, Malaysia
[6]Department of Medical Microbiology, Faculty of Medicine, University of Malaya, Lembah Pantai, 50603 Kuala Lumpur, Malaysia
*Corresponding author e-mail: Priya.Madhavan@taylors.edu.my
Received 27 June 2018; received in revised form 12 November 2018; accepted 12 November 2018

**Abstract.** The purpose of this study is to characterize 3 non-albicans *Candida* spp. that were collected from two major hospitals in a densely populated area of Kuala Lumpur for their susceptibilities to azole and genetic background. Fifteen non-albicans *Candida* clinical isolates in two major hospitals in Kuala Lumpur area of Malaysia were collected by convenience sampling during 2007 and 2010. The genetic diversity of 15 non-albicans *Candida* species comprising *C. glabrata* (*n* = 5), *C. parapsilosis* (*n* = 5) and *C. rugosa* (*n* = 5) were assessed by RAPD-PCR typing. Strains were initially identified using biochemical tests and CHROMagar *Candida* medium. Fluconazole and voriconazole susceptibilities were determined by E-test method. Commercial kits were used for DNA extraction and amplification with RAPD primers (OPA02, OPA03 and OPA08). PCR conditions were optimized and simultaneous identification was possible by agarose gel electrophoresis of PCR products and the bands obtained were analyzed using BioNumerics Applied Maths v.6.6 software. The RAPD primers used in this study generated 100% polymorphic profile. Cluster analysis using the RAPD-PCR profile showed 12.5-25% similarity among the strains. The genetic diversity was based on the strain susceptibility towards both the azoles, site of isolation and place according to their unique banding patterns. In contrast, strains susceptible to azoles were found to be genetically similar with clonal dissimilarity. The use of OPA02, OPA03 and OPA08 primers in differentiating non-albicans *Candida* spp. underscores the higher resolution of RAPD-PCR as a reliable tool for strain/species level differentiation.

## INTRODUCTION

*Candida* is the most common human fungal pathogen that has evolved genetic mechanisms resulting in a conspicuous shift in the trend of candidiasis aetiology.

The understanding of nosocomial infections requires a more discriminant method than species identification, such as molecular typing. Molecular typing methods can be used to identify the source i.e. by tracing the specific disseminated clone in an

environment. In addition, such typing methods are more reliable in the identification of reservoirs of infection or cross-contamination (Blanc, 2004). Some molecular typing methods use continuous marker values such as ribotyping, pulse-field electrophoresis (PFGE) and random amplified polymorphic DNA (RAPD), while those that use categorized values include multi-locus sequence typing (MLST). MLST uses several housekeeping genes with which differences in their sequences are assigned to alleles, providing an allelic profile of the isolates. However, this typing method is usually used to investigate long-term or global epidemiology with low discriminatory power and high workload compared to other methods. It has been noted that RAPD is more economical which provides fast and reproducible fingerprints with a comparable discriminatory level to PFGE (Bostock *et al.*, 1993). Recently RAPD-PCR typing method is increasingly being used to characterize both *C. albicans* and non-albicans *Candida* spp. (Malek *et al.*, 2017; Przybylowska *et al.*, 2017; Tapia *et al.*, 2017; Bertone *et al.*, 2016; Ying *et al.*, 2016). RAPD method has been successfully used to characterize several non-albicans *Candida* spp. including, *C. pelliculosa* (Lin *et al.*, 2013), *C. parapsilosis* into *C. metapsilosis* and *C. orthopsilosis* (del Pilar Vercher *et al.*, 2011) and *C. kefyr* (Kalkanci *et al.*, 2007). A recent study by Pires *et al.* (2013) showed the high significance of RAPD technique in identifying *C. albicans*, *C. tropicalis*, *C. dubliniensis* and *C. krusei* when compared with CHROMagar *Candida* for species identification. With respect to antibiogram/resistogram, distinct RAPD profiles were documented from fluconazole susceptible and resistant *C. albicans* strains recovered from AIDS patients (Jain *et al.*, 2001). In another study, OPE-03 primer was used to characterize 30 strains of *C. albicans* (Xu *et al.*, 2012). It is evident from these studies that *Candida* isolates could be characterized based on their drug susceptibilities using RAPD method. Therefore, the present study was aimed to characterize a limited collection of fluconazole-sensitive and fluconazole-resistant *Candida* spp. and voriconazole-sensitive and voriconazole-resistant *Candida* spp. using three 10-mers primers by RAPD-PCR method. Optimization was a crucial part in this study, hence we report the parameters used in the RAPD-PCR method.

## MATERIALS AND METHODS

### *Candida* isolates and culture conditions

Fifteen non-albicans *Candida* species comprising *C. glabrata* (*n* = 5), *C. parapsilosis* (*n* = 5) and *C. rugosa* (*n* = 5) collected from University Malaya Medical Centre (UMMC) and Gleneagles Intan Medical Centre (GIMC) in Kuala Lumpur were used in this study. *C. glabrata* was selected because of its resistance towards fluconazole, *C. parapsilosis* being the third most common cause of *Candida* infections worldwide and *C. rugosa* is a rare clinical isolate. The sources of these isolates were blood, high vaginal swab (HVS) and skin. Isolates were predominantly grown on CHROMagar Candida™ according to our previously described method (Madhavan *et al.*, 2011).

### MICs determination

Fluconazole and voriconazole MICs were determined using *E* test strips (AB Biodisk, Sweden) similar to the method described in our previous study (Madhavan *et al.*, 2010). Isolates were characterized based on their breakpoints for resistance according to the recommendations by Clinical and Laboratory Standards Institute (CLSI 2008).

### Strain identification using PCR

Total chromosomal DNA was extracted from the isolates using a innuPrep DNA mini kit (Analytikjena, Germany) according to the manufacturer's instructions. The DNA was quantified using a nanophotometer (Implen, Germany) and the purity of the DNA was set in the range of 1.7-2.0. PCR was performed using ITS 1 and 4 fungal primers to further confirm the identity of the isolates (Isogai *et al.*, 2010).

The reaction mixture of PCR was a $25 \mu L$ total volume containing of the $12.5 \mu L$ of master mix (EuRx, Gdansk, Poland), $2.5 \mu L$ of 10X PCR buffer, $2.5 \mu L$ of 25 mM $MgCl_2$, $0.5 \mu L$ of 10 $\mu M$ ITS 1 primer, $0.5 \mu L$ of 10 $\mu M$ ITS 4 primer, 1 $\mu L$ of 5 mM dNTPs and an appropriate amount of sterile distilled water. The master mix solution and 1 $\mu L$ of 100-150 ng DNA templates were transferred into each tube. Finally, $0.3 \mu L$ of 500 U *Taq* DNA polymerase was added to each tube. A negative control was prepared containing ultra-pure water replacing the DNA template. The PCR cycling was set on a Thermal Cycler C1000 (Bio-Rad, Singapore) under the following amplification conditions: initial denaturation at 94°C for 5 minutes, 25 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 35 seconds and extension at 72°C for 1 minute. The reactions were finalized by polymerization for 8 min at 72°C. The PCR amplicons were visualized using UV light AlphaImager® Gel Documentation after electrophoresis using 1.5% (w/v) agarose gel (Sigma-Aldrich) in 1X TBE containing $2.5 \mu L$ GelRed™ Nucleic Acid Gel Stain (Biotium, Inc., Hayward, CA, USA). The expected size for the amplicons of *C. glabrata* was around 880 bp, 500 bp for *C. parapsilosis* and 400 bp for *C. rugosa* (Isogai *et al.*, 2010; Mirhendi *et al.*, 2001).

The identities of the purified PCR products were confirmed by DNA sequencing which was performed by commercial sequencing service (First BASE Laboratories Sdn Bhd, Selangor, Malaysia). Computer-assisted sequence analysis of the genes was carried out using BLASTn and BLASTx at NCBI, Reverse Complement programme and ClustalW2 at The European Bioinformatics Institute (EMBL-EBI). The species was re-identified from the results obtained.

**Random amplification of polymorphic DNA (RAPD)**
Operon set A (Operon Technologies, Alameda, CA, USA) primers were used in this study to generate unique banding patterns for different *Candida* strains used. DNA samples were subjected to amplification using 3 decamers (OPA02, OPA03 and OPA08) primers (synthesized from Sigma Aldrich) with GC content varying from 60 to 80%. A range of other primers were also screened initially, however no bands were produced from these primers.

Optimization of PCR conditions for best banding patterns was a crucial part of this study. Amplification reactions were carried out with the aforementioned oligonucleotide primers. The optimized PCR cycling conditions were as follows: initial denaturation at 95°C for 5 min, 45 cycles of denaturation at 94°C for 45 sec, annealing at 45°C (OPA02 and OPA03) and 50°C (OPA08) for 45 sec and extension at 73°C for 45 sec, followed by final extension at 72°C for 5 min. DNA was extracted according to the method described in section 2.3. The standard optimized PCR was carried out in a 30 $\mu L$ volume containing 0.7 $\mu L$ of DNA (150-200 ng), 3 $\mu L$ of 10X buffer, 2.4 $\mu L$ of 25 mM $MgCl_2$, 2.6 $\mu L$ of 10 mM dNTPs, 0.6 $\mu L$ of OPA primer, 20.2 $\mu L$ sterile $dH_2O$ or ultrapure water and 0.5 $\mu L$ of *Taq* DNA polymerase (EuRx, Gdansk, Poland). PCR products were resolved along with a 100 bp molecular weight marker (New England Biolabs, UK) visualized using UV light AlphaImager® Gel Documentation after electrophoresis on 2.5% (w/v) agarose gel (Sigma-Aldrich) in 1X TBE containing $2.5 \mu L$ GelRed™ Nucleic Acid Gel Stain (Biotium, Inc., Hayward, CA, USA). The DNA profiles of the strains were characterized based on their DNA banding patterns using the BioNumerics programme (Version 6.6, Applied Maths) and a phylogenetic tree was developed clustering similar species together. The analysis was carried out with Dice coefficient correlation, followed by UPGMA (unweighted pair group method with arithmetic mean) cluster analysis (Grundmann *et al.*, 1997). Dice coefficient is based on the presence or absence of bands in particular positions. Identical strains were clustered at 100%, highly similar but not identical strains were clustered between 70% and 99%, whereas unrelated strains were clustered at below 70%.

## RESULTS AND DISCUSSION

All the *Candida* species isolated from blood were collected at UMMC. Besides blood as the main source of isolation, one *C. rugosa* isolate (Cr5) was from skin, two *C. parapsilosis* strains and one *C. glabrata* isolate (Cg5) was from HVS. In GIMC, there were two *C. parapsilosis* isolates from HVS and a *C. glabrata* isolate (Cg6) was from skin. In terms of their susceptibilities towards fluconazole and voriconazole, 4 *C. rugosa* isolates were susceptible to both azoles and one *C. rugosa* isolate (Cr3) with a reduced susceptibility (SDD) towards fluconazole (all from UMMC). Among *C. parapsilosis*, 4 isolates from HVS were susceptible to both azoles (2 from UMMC and 2 from GIMC). However, there was one invasive isolate (blood) from UMMC that showed susceptibility (SDD) towards fluconazole. Among the *C. glabrata* isolates, 3 invasive strains from UMMC were susceptible to both azoles, one HVS isolate from UMMC was resistant to both azoles and one skin isolate from GIMC was resistant to fluconazole only. Therefore, our objective was achieved in this study to distinguish the susceptible, SDD and resistant strains using RAPD according to their unique banding patterns. In contrast, even the susceptible isolates showed a high similarity but not clonally related, which indicates that they have different genotypes and require further investigations.

RAPD fingerprints of the 15 clinical isolates including the 3 ATCC strains were obtained. RAPD profiles using primers OPA02, OPA03 and OPA08 were shown in Fig. 1, Fig. 2 & Fig. 3. In a study conducted using *Candida* isolates from Cancer patients in Iran, a low annealing temperature of 36°C showed better identity of the bands, and up to 5 *Candida* species were successfully identified using a single primer (Saltanpour *et al.*, 2011). The bands analyzed using the BioNumerics Applied Maths v.6.6 software resulted in a composite dendrogram as shown in Fig. 4.

Of the total 15 *Candida* isolates, 3 isolates were from GIMC while 12 isolates were from UMMC. The source of these isolates were as follows: 8 isolates were from blood, 5 from HVS and 2 from skin. Isolates obtained from GIMC included *C. glabrata* and *C. parapsilosis*, while UMMC isolates contained all three species. The bands for all isolates were detected between 100 bp and 3000 bp. The isolates were genotyped and grouped into 3 major groups according to their species using composite DNA type (based on all three primers). The first group consisted of *C. rugosa* strains, followed by *C. parapsilosis* and *C. glabrata* as second and third group respectively.

Cluster analysis of *C. rugosa* strains showed that isolates Cr2, Cr4 and Cr6 belonged to the same cluster with 86% similarity. Invasive *C. rugosa* isolates (Cr2 and Cr4) from the same hospital, but on different years (2007 and 2010) showed 92% similarity. While cluster analysis of isolates Cr1 and Cr3 from blood and Cr5 from skin were found to be genetically dissimilar. In this study, all *C. rugosa* isolates were from a single hospital (UMMC), and the genetic relatedness of *C. rugosa* isolates from GIMC is unknown due to lack of samples. Cluster analysis of *C. parapsilosis* isolates showed that isolates Cp3 and Cp4 were clustered together. These two isolates were recovered from HVS in the same year from UMMC and further analysis revealed that Cp3 and Cp4 were genetically similar with clonal dissimilarity. Meanwhile, isolates Cp5 and Cp6 recovered from HVS from GIMC were found to be genetically dissimilar. Invasive isolate Cp2 from blood showed a reduced susceptibility (SDD) towards fluconazole which gave rise to another genotype in our *C. parapsilosis* collection.

*C. glabrata* isolates Cg2, Cg3 and Cg4 belonged to the same lineage which was clustered together at 74%. These 3 isolates displayed similar susceptibilities towards fluconazole and voriconazole (Table 1 and Fig. 4). Isolates Cg3 and Cg4 were found to have similar phenotypic features but of different clones were clustered together and these isolates had 82% similarity. The sources of *C. glabrata* isolates Cg1, Cg5 and Cg6 were different and cluster analysis of these isolates showed that the isolates were genetically dissimilar.
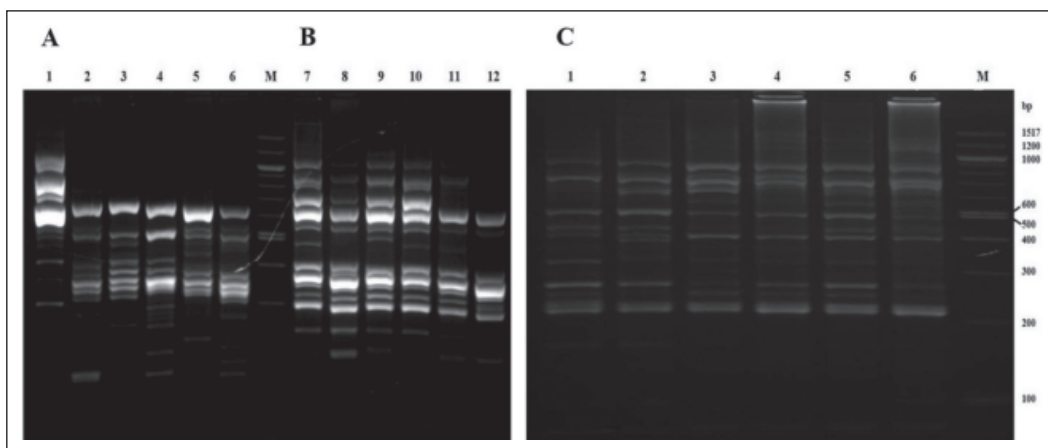
Figure 1. RAPD profile of the studied *C. glabrata* strains using (**A**) OPA03 and (**B**) OPA02 (**C**) OPA08 primers. *Lane 1* - Cg ATCC, *Lane 2* - Cg 1, *Lane 3* - Cg 2, *Lane 4* - Cg 3, *Lane 5* - Cg 4, *Lane 6* - Cg 5, M - 100 bp molecular weight marker.
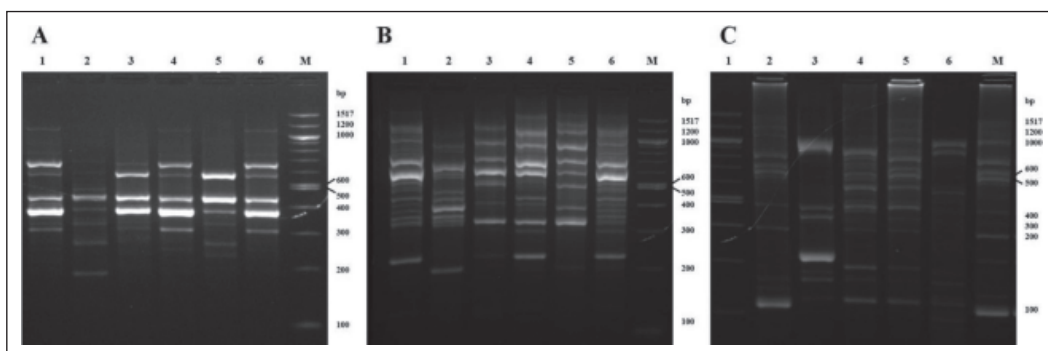


Figure 2. RAPD profile of the studied *C. parapsilosis* strains using (**A**) OPA02 and (**B**) OPA03 (**C**) OPA08 primers. *Lane 1* - Cp ATCC, *Lane 2* - Cp 1, *Lane 3* - Cp 2, *Lane 4* - Cp 3, *Lane 5* - Cp 4, *Lane 6* - Cp 5, M - 100 bp molecular weight marker.
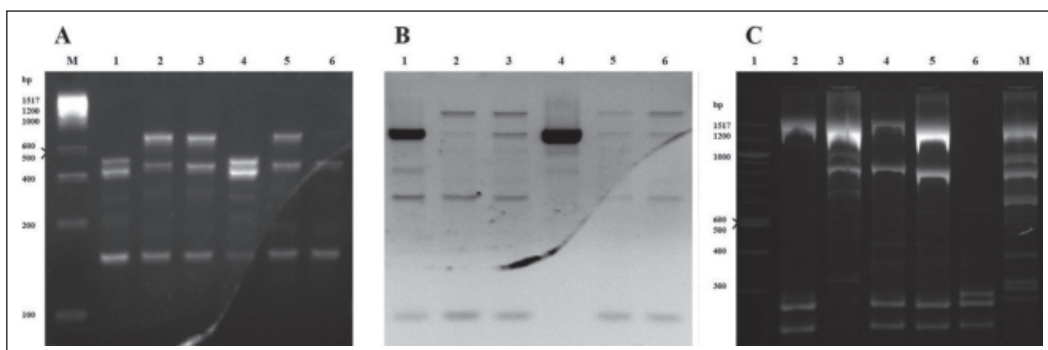


Figure 3. RAPD profile of the studied *C. rugosa* strains using (**A**) OPA02 and (**B**) OPA03 (**C**) OPA08 primers. *Lane 1* - Cr ATCC, *Lane 2* - Cr 1, *Lane 3* - Cr 2, *Lane 4* - Cr 3, *Lane 5* - Cr 4, *Lane 6* - Cr 5, M - 100 bp molecular weight marker.

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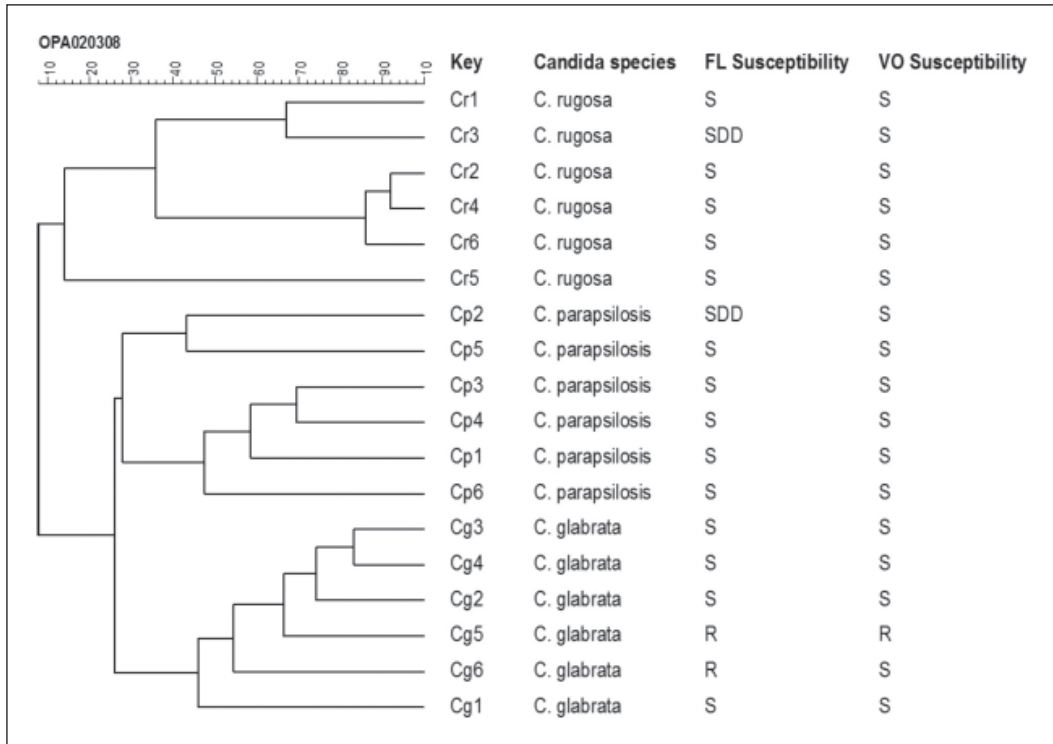| Key | Candida species | FL Susceptibility | VO Susceptibility |
|---|---|---|---|
| Cr1 | C. rugosa | S | S |
| Cr3 | C. rugosa | SDD | S |
| Cr2 | C. rugosa | S | S |
| Cr4 | C. rugosa | S | S |
| Cr6 | C. rugosa | S | S |
| Cr5 | C. rugosa | S | S |
| Cp2 | C. parapsilosis | SDD | S |
| Cp5 | C. parapsilosis | S | S |
| Cp3 | C. parapsilosis | S | S |
| Cp4 | C. parapsilosis | S | S |
| Cp1 | C. parapsilosis | S | S |
| Cp6 | C. parapsilosis | S | S |
| Cg3 | C. glabrata | S | S |
| Cg4 | C. glabrata | S | S |
| Cg2 | C. glabrata | S | S |
| Cg5 | C. glabrata | R | R |
| Cg6 | C. glabrata | R | S |
| Cg1 | C. glabrata | S | S |

Figure 4. Dendrogram generated using RAPD profile of the studied *Candida* strains. Dendrogram was constructed following the UPGMA method.

Table 1. Clinical strains of *Candida* species and susceptibility to azoles

| *Candida* species and strain number | $MIC_{80}$ / Susceptibility to fluconazole | $MIC_{80}$ / Susceptibility to voriconazole | Place/Year of isolation | Site of isolation |
|---|---|---|---|---|
| *C. rugosa* 1 (ATCC 10571) | 0.5/Susceptible | 0.004/Susceptible | NA | NA |
| *C. rugosa* 2 | 8/Susceptible | 0.064/Susceptible | UMMC/2010 | Blood |
| *C. rugosa* 3 | 12/SDD | 0.023/Susceptible | UMMC/2007 | Blood |
| *C. rugosa* 4 | 8/Susceptible | 0.064/Susceptible | UMMC/2007 | Blood |
| *C. rugosa* 5 | 8/Susceptible | 0.047/Susceptible | UMMC/2007 | Skin |
| *C. rugosa* 6 | 8/Susceptible | 0.064/Susceptible | UMMC/2007 | Blood |
| *C. parapsilosis* 1 (ATCC 22019) | 1.5/Susceptible | 0.023/Susceptible | NA | NA |
| *C. parapsilosis* 2 | 12/SDD | 0.064/Susceptible | UMMC/2007 | Blood |
| *C. parapsilosis* 3 | 0.5/Susceptible | 0.004/Susceptible | UMMC/2007 | HVS |
| *C. parapsilosis* 4 | 0.5/Susceptible | 0.012/Susceptible | UMMC/2007 | HVS |
| *C. parapsilosis* 5 | 0.25/Susceptible | 0.023/Susceptible | GIMC/2008 | HVS |
| *C. parapsilosis* 6 | 0.25/Susceptible | 0.064/Susceptible | GIMC/2008 | HVS |
| *C. glabrata* 1 (ATCC 2001) | 2/Susceptible | 0.064/Susceptible | NA | NA |
| *C. glabrata* 2 | 1.5/Susceptible | 0.094/Susceptible | UMMC/2010 | Blood |
| *C. glabrata* 3 | 2/Susceptible | 0.19/Susceptible | UMMC/2010 | Blood |
| *C. glabrata* 4 | 1.5/Susceptible | 0.094/Susceptible | UMMC/2007 | Blood |
| *C. glabrata* 5 | > 256/Resistant | 6/Resistant | UMMC/2007 | HVS |
| *C. glabrata* 6 | 96/Resistant | 0.19/Susceptible | GIMC/2008 | Skin |

ATCC - American Type Culture Collection; UMMC - University Malaya Medical Centre; GIMC - Gleneagles Intan Medical Centre; HVS - High vaginal swab; NA - not applicable.

RAPD generates complex patterns that vary among unrelated strains. Although RAPD was deprived for its low reproducibility between laboratories, it is still a widely used technique to study the epidemiology of several infectious pathogens, including *Candida* (Lin *et al.*, 2013; Gyanchandani *et al.*, 1998; Robert *et al.*, 1995). The use of 10-mer primers is also an added advantage as there are numerous 10-mer primers available when compared to enzyme-based systems (Marais *et al.*, 2004). The use of a single primer in differentiating *Candida* strains has been reported adequately (del Pilar Vercher *et al.*, 2011; Xu *et al.*, 2012; Saltanpour *et al.*, 2011). Despite the moderate discriminatory power of RAPD-PCR technique, the use of different primers in independent runs and combining the data for analysis increases its discriminatory power (Pujol *et al.*, 1997). This is known as composite DNA typing with which microevolution between strains can be detected. This is one of the main reasons for us to use 3 primers in order to obtain a higher discriminatory power. In another study, 2 decamers were used in RAPD-PCR to characterize 14 *Candida* isolates and was found a better method compared to CHROMagar *Candida* medium (Imran *et al.*, 2014). Similarly, five random decamers were used in RAPD to characterize *Candida* species in an intensive care unit in Poland (Paluchowska *et al.*, 2014).

The limited sample size and the lack of clinical data to correlate drug susceptibility pattern, as well as the determination of clonal types in each hospital are some of the limitations of this study. In the management of recurrent *Candida* infections, proper patient demography is crucial in correlating the laboratory findings (Chong *et al.*, 2007). This, in fact, provides information on the antibiotics used and details of recurrent infections in the same patient. Vulvovaginal candidiasis is known to be the second most frequent genital tract infection among females (Sobel, 2005). In this study, 33% of the isolates were recovered from HVS, however, the patients' demography and medical condition are not known which makes it difficult to identify the predisposing factors related to the strain susceptibilities towards fluconazole and voriconazole.

Despite the low interlaboratory reproducibility of RAPD technique, it can be used to investigate local outbreaks (Saghrouni *et al.*, 2013). The use of RAPD-PCR method has identified the source of contamination from particular work stations where it was prepared (Marais *et al.*, 2004). In addition, the source of infections can be determined by molecular typing methods, especially incidences of *C. parapsilosis* outbreaks in neonatal intensive care unit (NICU) (Reissa *et al.*, 2008) and systemic infections due to *C. parapsilosis* (Paluchowska *et al.*, 2014). The use of central intravascular catheters, prior exposure to haemodialysis or antibiotics remain to be the main cause of systemic infections (Binelli *et al.*, 2006; Pittet *et al.*, 1994). In particular, catheter tips were reported to be the main reservoir of *C. parapsilosis* in majority of the candidaemia cases. Blood cultures positive for invasive *Candida* isolates accounted for 53% of the total number of isolates (Miranda *et al.*, 2009).

According to the ARTEMIS DISK antifungal surveillance program conducted across 127 different medical centers worldwide in 2010, it is evident that 0.4% of *Candida* spp. belong to *C. rugosa* (Pfaller *et al.*, 2010). Previously, genetic heterogeneity among *C. rugosa* isolates has been reported previously in which the isolates were clustered into 9 groups. In this study, *C. rugosa* strains were clustered into four distinct groups (Redkar *et al.*, 1996). Reports of *C. rugosa* on catheters and total parenteral nutrition (TPN) suggests that species identification and strain characterization is important in providing better treatment to patients with *Candida* infections (Minces *et al.*, 2009). The strains studied herein were randomly compiled and has not been formally aimed for an epidemiological investigation. However, this technique could be used to study the actual source of *C. rugosa* infection and its microevolution in centers where such infections are common.

This study also did not detect any clustering of a dominant strain which indicates that the microevolution of these polyclonal strains had occurred from a previously dominant strain. Previously, substrain shuffling has been proposed in women with recurrent *Candida* vaginitis (Lockhart *et al.*, 1996). In this event, minor genetic variations are possible in recurrent infections among patients with vaginitis, suggesting substrain shuffling between recurrences. The same mechanism could have been responsible in this study for the disparity observed within strains studied. Several other studies have also suggested that micro evolutionary changes in a single strain during its adaptation to environmental conditions (Bonfim-Mendonca *et al.*, 2013; Soll, 2007). Molecular markers are not only advantageous for the characterization of strains but also in identifying the parent cells (Hashoosh *et al.*, 2015).

Our results show that it is possible to use RAPD in identifying and differentiating major species of *Candida*. The choice of using OPA02, OPA03 and OPA08 primers was good in distinguishing *Candida* strains. However, to achieve higher discriminatory power, more number of primers should be used. The experimental approach of RAPD-PCR can lead to the development of specific probes to identify specific clonal types. In order to achieve this, a larger number of samples are required so that the diagnostic bands can be identified.

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