Multilocus sequence typing of *Candida albicans* isolates from a burn intensive care unit in Iran

Mohammad H. Afsarian,1,2 Hamid Badali,1 Teun Boekhout,3 Tahereh Shokohi1 and Farzad Katiraee4

1Department of Medical Mycology and Parasitology/Invasive Fungi Research Center (IFRC), School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran
2Department of Microbiology, Fasa University of Medical Sciences, Fasa, Iran
3CBS Fungal Biodiversity Centre (CBS-KNAW), Utrecht, The Netherlands
4Department of Pathobiology, Division of Mycology, School of Veterinary Medicine, University of Tabriz, Tabriz, Iran

Burn intensive care unit (BICU) patients are specifically exposed to deep-seated nosocomial infections due to *Candida albicans*. Superficial carriage of *C. albicans* is a potential source of infection and dissemination, and typing methods could be useful to trace the different isolates. Multilocus sequence typing is a powerful genotyping method for pathogenic micro-organisms, including *Candida albicans*. Thirty clinical isolates of *C. albicans* obtained from 22 patients that were admitted to the BICU from a burn hospital at Sari, Mazandaran state, Iran, were studied epidemiologically by multilocus sequence typing (MLST). Seventy-five variable nucleotide sites were found. Sixty-two alleles were identified among the seven loci of the *C. albicans* isolates and one new allele was obtained. Eighteen diploid sequence types (DSTs) were identified, and among those 10 were new. These isolates belonged to nine clonal clusters (CCs) while two isolates occurred as singletons. Eleven (36.7 %) isolates belonged to CC 124 after eBURST analysis and 13 isolates (43.3 %) were assigned to clade 4. Approximately 17 % of the 30 isolates belonged to clade 1 (CC 69 and CC 766). Isolates from several patients with burns were found to be related genetically. Some patients yielded multiple isolates with identical DSTs, suggesting colonization or infection caused by cross-contamination between patients. Isolates that show identical or similar allelic profiles are presumed to be identical or closely related and may be used to evaluate the genetic relationships between isolates from a specific environment such as the BICU.

**INTRODUCTION**

Fungal infections due to *Candida* species have increased during recent decades as these yeasts have emerged as an important opportunistic fungal pathogen. *Candida* species can cause various clinical manifestations ranging from superficial to systemic candidiasis in either immunocompetent or immunocompromised hosts (Garcia-Hermoso et al., 2007; Odds et al., 2007; Pfaller & Diekema 2007; Fesharaki et al., 2013). Infections due to *C. albicans* are an important cause of morbidity and mortality among hospitalized patients who have been admitted to intensive care units (ICUs) and in oncology wards worldwide (Bougnoux et al., 2006, 2008; Horn et al., 2009; Tortorano et al., 2012; Sardi et al., 2013). Burn patients are especially susceptible to *Candida* infections because they have most of the risk factors for hospital-acquired fungal infections, i.e. cutaneous and vascular portals of entry (inhabiting catheters, parenteral nutrition) and broad-spectrum antimicrobial therapy. In most cases, colonization by *C. albicans* of the skin or mucosa occurs before invasion and systemic infection, and colonization may be a major reservoir for cross-contamination. Therefore, identification of the source of colonization and the transmission route of infection will contribute to the prevention of nosocomial infections due to *C. albicans* among burn patients (Bougnoux et al., 2004; Lee et al., 2013). Nosocomial candidiasis caused by *C. albicans* requires a precise understanding of the epidemiology in different geographical regions using a reliable typing system. Recently, several molecular typing techniques have been used to study epidemiological relationships of *C. albicans* isolates such as randomly amplified polymorphic DNA (Robert et al., 1995), RFLP (Vazquez et al., 1993), Southern blot hybridization with discriminating probes (Myyong et al., 2011), amplified fragment length polymorphism analysis (Borst et al., 2003) and microsatellites length
polymorphism (Garcia-Hermoso et al., 2007). The multi-locus sequence typing (MLST) technique has been used extensively because it yields high levels of resolution allowing it to characterize a large number of isolates rapidly, and it does not require any subjective interpretation of banding patterns. Moreover, MLST sequences can be stored and characterized in multiple electronic formats, thus offering an unprecedented degree of portability and accessibility to other users (website http://calbicans.mlst.net) (Bougnoux et al., 2003, 2004). The purpose of this study was to investigate the MLST analysis of clinical isolates of C. albicans recovered from patients with burns in a burn intensive care unit (BICU) of a hospital at Sari city, Iran. To our knowledge, it is also the first nationwide study into the genotypic relationships of C. albicans isolates from Iran using the MLST method.

**METHODS**

Thirty clinical isolates of C. albicans were collected from hospitalized patients at Sari city, Iran. These strains were isolated from 22 burn patients with superficial colonization or candidiasis, candiduria and candidaemia that were admitted to the BICU of the burn hospital from July 2011 to March 2012. The skin burns were sampled using sterile swabs (Becton Dickinson) and samples cultured onto Sabouraud’s dextrose agar (SDA; Difco) medium supplemented with chloramphenicol (SC). Blood samples were inoculated onto biphasic fungal blood culture media containing brain heart infusion (BHI; Difco) broth and BHI agar, and incubated for 10 days at 37 °C. Blood cultures were subcultured on SC and CHROMagar Candida (bioMérieux). Five patients were sampled at different body sites and different times: skin burns (hand), blood and urine of patient 2 (P2) were sampled within a 7 day period; skin burns (hand and abdomen), blood and urine of P3 were sampled within a 10 day period; skin burns (foot) and urine of P7 were sampled within a 7 day period; skin burns of P13 including chest and scapula were sampled within a 5 day period; skin burns (chest) and two blood samples of P17 were sampled within a 21 day period. MLST was used to type the 30 clinical isolates as described previously (Bougnoux et al., 2003). Briefly, genomic DNA was extracted using glass beads and the phenol/chloroform method (Yamada et al., 2002) and stored at −20 °C prior to use. MLST used seven housekeeping genes, AAC1, ACC1, ADP1, MPIb, SYA1, VPS13 and ZWF1b (Bougnoux et al., 2003). PCR amplification was performed for seven loci using a thermal cycler (Bio-Rad-C1000) and previously described primers (Bougnoux et al., 2003), followed by sequencing using an ABI 3730XL automatic sequencer (Applied Biosystems, Foster City, CA, U.S.A.). Sequence data were aligned manually using MEGA 5.05 (http://www.megasoftware.net) (Tamura et al., 2011) and BioEdit version 7.0.9 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) (Alignment, BioEdit Sequence 2011) software packages. Heterozygosity was identified by the presence of two peaks on both strands at the same loci and the consensus sequences for the seven loci of all isolates were defined.

The number of alleles and diploid sequence types (DSTs) was determined by comparing the sequences with those available in the C. albicans MLST database (http://calbicans.mlst.net). Chromatograms for the isolates with new alleles and new DSTs were sent to the central MLST database curator and new MLST numbers were assigned. Newly identified DSTs were assigned the numbers 2240 to 2251 (except 2246 and 2248) in http://calbicans.mlst.net/sql/burstspadvanced.asp. Epidemiological relationships were assessed by eBURST analysis (http://eburst.mlst.net/) (Feil et al., 2004) and DSTs of 30 isolates were compared with those available (n=2099) in the MLST database in June 2014. This algorithm places all related isolates into groups called clonal clusters (CC: set of DSTs that are believed to have descended from the same founding genotype). CCs were identified using a reference DST for each clade according to previously described major C. albicans clades (Odds et al., 2007; Odds, 2010). CCs of isolates differ in sequence at only one of the seven loci (Odds et al., 2007). The unweighted pair group method with arithmetic mean (UPGMA) dendrogram based on MLST sequence data was drawn using CLC Sequence Viewer 6 software (http://www.clcbio.com/).

**RESULTS**

Thirty epidemiologically related isolates of C. albicans were obtained from 22 burn patients (age range: 2–60 years) admitted to the BICU. C. albicans strains were isolated from blood cultures (n=6), skin burns (n=19), urine (n=3), catheter (n=1) and sputum (n=1). The MLST data of the 30 C. albicans isolates consisted of 2883 nt. Seventy-five (2.6 %) nucleotide sites were found to be variable and heterozygous in at least one isolate. Thus the seven genes sequenced yielded a total of 75 variable sites of which VPS13 produced the highest number (n=14) of polymorphic sites, while ACC1 produced the lowest number (n=6). Sixty-two alleles were identified in the seven loci studied. The gene VPS13 generated the highest number of alleles (n=11), while ACC1 generated the lowest number (n=7). Among the alleles, one new allele was determined in the ADP1 locus (allelic number 122) and this was added to the MLST database (http://calbicans.mlst.net). Dated June 2014, from the 125 alleles present only allele number 122 of the ADP1 locus displayed a polymorphism at nucleotide site 366 (Y instead of C).

Eighteen unique DSTs were obtained in the seven loci of the 30 Iranian C. albicans isolates from 22 BICU patients. Each allelic profile query has been submitted to http://www.calbicans.mlst.net. Eight of these 18 DSTs had been identified previously and 10 novel DSTs were added to the online database (http://calbicans.mlst.net). The eBURST program was used for the analysis of the genotypic relationships among the MLST data of the 30 Iranian C. albicans strains using all available DSTs (n=2099) in the MLST database. This yielded 108 eBURST groups (CCs) and 707 singleton isolates. The 30 isolates in this study were placed in 9 CCs while two isolates (DST 2095) were singletons. Eleven Iranian isolates, i.e. 36.7 %, recovered from eight patients belonged to CC 124, four isolates (13.3 %) from four patients belonged to CC 172, three isolates (10 %) from two patients belonged to CC 69, two isolates (6.7 %) from two patients belonged to CC 918, three isolates (10 %) from one patient belonged to CC 601, two isolates (6.7 %) belonged to CC 461, one isolate (3.3 %) belonged to CC 1950 and one from two patients belonged to CC 766, one isolate (3.3 %) belonged to CC 461 and one isolate (3.3 %) belonged to CC 1865. However, according to the latest classification by Odds et al. (2007) and Odds (2010), the taxonomical position for new DSTs (2099 and 2102) has not been clarified so far (Fig. 1).

Among 30 isolates of C. albicans collected from skin burns and blood cultures of 22 patients in the same BICU, the
MLST analysis showed that some isolates had the same DST (Fig. 1). From 22 patients in this study, 19 isolates from skin burns or cutaneous candidiasis yielded 13 DSTs belonging to four clades (1, 4, 12 and 15), of which seven DSTs belonged to clade 4 because some alleles were shared between seven DSTs. These results may suggest the presence of microvariation due to microevolution of the genomes of epidemiologically related strains of *C. albicans* in the BICU (Fig. 1). Three out of the seven strains isolated from candidaemic patients with burns belonged to clade 4 (CC 124) and two of them originated from multiple episodes of one patient (P17). Four other strains were assigned to four CCs (69, 918, 461 and 601) in four clades (1, 9, 11 and 12). Moreover, four isolates recovered from candidaemic patients were identified as new DSTs (Fig. 1).

Some patients yielded multiple isolates with identical DSTs, four patients (P4, P9, P10 and P12) yielded isolates identified as DST 172, two patients (P19 and P22) had DST 656, two patients (P5 and P13) had DST 124 and one patient (P3) was infected with *C. albicans* isolates belonging to two DSTs (i.e. 918 and 2093) (Table 1). Five patients (P2, P3, P7, P13 and P17) were sampled at different body sites (Table 1), of which four patients appeared to harbour just one strain of *C. albicans*, whereas one patient (P3) yielded two different strains isolated from three samples (blood, urine and skin burns) (Table 1).

**DISCUSSION**

MLST is a powerful and useful technique for understanding the epidemiological and evolutionary relationships of pathogenic micro-organisms (Taylor & Fisher, 2003; Urwin & Maiden, 2003; Maiden, 2006; Cliff *et al.*, 2008; Odds & Jacobsen, 2008; Da Matta *et al.*, 2010; Shin *et al.*, 2011). Prevalence of *C. albicans* infections in the BICU of the Zare hospital (Sari, Iran) led us to investigate the epidemiological relationship of 30 isolates involved using the MLST method. Although the sample size of strains...
typed herein was low, our study revealed 10 new DSTs, i.e. 33.3%, among 30 isolates. Three new DSTs belonged to clade 4 (CC 124), as did a new singleton DST. The remainder of the isolates belonged to various CCs. Twenty-eight isolates were found to be located in nine CCs by eBURST analysis and two isolates were identified as singletons, thus revealing a high genetic heterogeneity among the \textit{C. albicans} population of these hospitalized patients.

Among a large assembly of strains collected worldwide, Odds \textit{et al.} (2007) and Odds (2010) showed that \textit{C. albicans} clade 1 (CC 69) was the most prevalent one followed by clade 4 (CC 124), clade 3 (CC 344), clade 2 (CC 155) and clade 11 (CC 538), respectively. In addition, they found that 34\% of the Middle East isolates belonged to clade 1. Ninety-three per cent of our isolates clustered within 6 of the 17 known clades and 63\% belonged to three of the five major clades (i.e. clades 1, 4 and 11) as defined previously (Odds, 2010). In contrast, approximately 17\% of our isolates belonged to clade 1 including CC 69 and CC 766, whereas 43\% belonged to clade 4 including CC 124. Alastruey-Izquierdo \textit{et al.} (2013) identified 37 DSTs of \textit{C. albicans} including 17 new DSTs from Israel and they concluded that most strains belonged to CC 124 in both candidaemia and superficial candidiasis isolates. Notably none of our strains clustered in clade 2 (CC 155) and clade 3 (CC 344). In contrast, Gammelsrud \textit{et al.} (2012) analysed 62 strains from Norway and found 32 DSTs of which 31\% belonged to clade 2 (CC 155). Shin \textit{et al.} (2011) found 58\% new DSTs among 156 Korean isolates, and 15\% of the isolates clustered in clade 4 and 19\% clustered in a new Asia-specific clade with $p$-distance $\leq 0.035$, while based on the $p$-distance $\leq 0.04$ (Odds, 2010), all isolates were assigned to clade 3. In the present study, our results showed that none of the Iranian isolates belonged to this Asia-specific clade.

Isolates obtained from different body sites of patients belonged to the same CC, for example P2, P7, P13 and P17 (Fig. 1). This finding is in accordance with previous studies that reported that \textit{C. albicans} strains isolated from different body sites of a person are usually genetically similar or identical (Bougnoux \textit{et al.}, 2006; Cliff \textit{et al.}, 2008), whereas two strains from P3 were assigned to two CCs, 69 and 918. Some DSTs (656, 124 and 172) of \textit{C. albicans} were isolated from more than one patient in the same BICU during one month; therefore, it seems that colonized patients are a major reservoir of \textit{C. albicans} in hospitals, and the isolation of \textit{C. albicans} with the same DST in more than one patient may provide evidence for cross-contamination between patients, which might be a crucial factor for nosocomial infections (Bougnoux \textit{et al.}, 2003, 2004, 2006).

Several closely related strains were isolated from patients. For example, the new DST 2093 differs from DST 69 only in locus \textit{ADP1} with one nucleotide different that may have arisen from a single recombination event. Similarly DST 2101 differs from DST 766 only in the locus \textit{ADP1} with one nucleotide different and these four DSTs were assigned to
clade 1 (Fig. 1). Moreover three new DSTs, including 2092, 2096 and 2103, assigned to CC 124, were found to be different in three loci from DSTs 124, 605 and 656 (Fig. 1). These findings may suggest micro-evolutionary changes of a single strain occurring during adaptation to varying environmental conditions (Bonfim-Mendonça et al., 2013; Paluchowska et al., 2014).

Cliff and coworkers showed evidence for the presence in an ICU of an endemic strain that was isolated repeatedly from patients and staff, and suggested horizontal transmission of C. albicans in the unit (Cliff et al., 2008). Bougnoux and colleagues using the MLST technique showed frequent colonization of C. albicans in a person or in the whole family by genetically closely related isolates that differed at one or several of the MLST loci (Bougnoux et al., 2006).

In conclusion, although a limited number of isolates were analysed in the current study, a significant genetic relationship was identified among epidemiologically related C. albicans isolates from burn patients in Iran suggesting a likelihood of nosocomial spread. Isolates that show identical or highly similar allelic profiles are presumed to be identical or closely related and may be used to evaluate the genetic relationships between isolates from a specific environment such as a BICU. To the best of our knowledge, this study is the first MLST study analysing C. albicans clinical isolates from Iran, thus also improving the epidemiological knowledge of C. albicans in this region.

ACKNOWLEDGEMENTS

This research was supported financially by a grant from the Mazandaran University of Medical Science (no. 91-32), which we gratefully acknowledge. We would like to thank Nazanin Lotfi and Seyede Zahra Nouranibaladezaei for technical assistance. We acknowledge the use of the Candida albicans MLST database, which is located at Imperial College London and is funded by the Welcome Trust. We also thank the curator of the MLST database, Marie-Elisabeth Bougnoux, for assigning our new alleles and DSTs to the C. albicans MLST database. T. B. was supported by a grant from the Qatar National Research Fund (HPRP 5-298-3-086), a member of Qatar Foundation. The statements herein are solely the responsibility of the authors.

REFERENCES


