Iran J Public Health, Vol. 44, No.9, Sep 2015, pp.1262-1269

Original Article

Molecular Diversity of *Candida albicans* Isolated from Immunocompromised Patients, Based on MLST Method

Seyed Mohammad Hosein AFSARIAN [1, 2], ***Hamid BADALI*** [2, 3], ****Tahereh SHOKOHI*** [2], ***Sohrab NAJAFIPOUR*** [1]

1. *Dept. of Microbiology, Fasa University of Medical Sciences, Fasa, Iran*
2. *Invasive Fungi Research Center (IFRC)/Dept. of Medical Mycology and Parasitology, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran*
3. *Molecular and Cell Biology Research Center, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran*

***Corresponding Author:** Email: Shokohi.tahereh@gmail.com

(Received 08 Jan 2015; accepted 24 Jun 2015)

Abstract

Background: As regards multilocus sequence typing (MLST) method directly analyze the polymorphism within DNA sequences; we performed the first nationwide study on the genotypic relationships of *Candida albicans* strains obtained from oropharynx and bronchoalveolar lavage (BAL) samples from immunocompromised patients.

Methods: Fourteen epidemiologically unrelated clinical strains of *C. albicans* were obtained from three hospitals in Mazandaran Province, Iran (2006 to 2012) from seven patients with pulmonary infections and the rest with oropharyngeal samples of immunocompromised patients. Seven loci of housekeeping genes were sequenced for all fourteen isolates.

Results: MLST was applied to a subset of 14 unrelated isolates. Seventy-one (2.5%) nucleotide sites were found to be variable. Accordingly, 60 different alleles were identified in seven loci among the isolates, among which two new alleles were obtained. Furthermore, 12 independent diploid sequence types (DSTs) including five novel DSTs were identified. The fourteen unrelated isolates were placed in 10 clonal clusters (CC) while two isolates were singletons, by eBURST analysis. Most of the isolates belonged to CC461 of eBURST analysis from the clade 11 and two isolates assigned to CC172 from the clade 15.

Conclusion: Pathogen distribution and relatedness for determining the epidemiology of nosocomial infections is highly recommended for pathogen control methods.

Keywords: MLST, *Candida albicans,* Genetic diversity, Unrelated strains

Introduction

Nowadays, the incidence of fungal agents causing candidiasis has increased remarkably, especially in patients infected with human immunodeficiency virus (HIV), immunocompromised hosts, intravenous drug abusers, bone marrow and solid organ recipient, those who present underlying valvular heart diseases, implantation of prosthetic valves and prolonged use of intravenous catheters. *Candida albicans* is one of the most prevalent opportunistic agent in candidiasis specifically in immunocompromised host followed by non *albicans* (1-4), however, approximately 17% of the nosocomial infections are caused by *C. parapsilosis*, *C. glabrata*, *C. tropicalis* and *C. krusei.* Moreover, critically ill patients admitted to the oncology and infectious disease wards are at greatest risk of fungal infections (5-9). Colonized patients are the main reservoir of *C. albicans* in hospitals, and the cross contamination may occurs between them. Thus, understanding the source of colonization and the transmission route, to control of

nosocomial infections due to *C. albicans* among admitted patients will improve, and recognize the characteristics of the infectious strains (10-12).

Due to the high degree of phenotypic similarity between *Candida* species, identification and differentiation may not be very successful, are time consuming and difficult to interpret. Therefore, it is now well established that advanced molecular methods, which have driven new developments in fungal taxonomy, are more reliable than classical methods (13-15). MLST is one of a series of techniques for phylogenetic studies and genotyping and is well suited for distinguishing closely related organisms at the species to strain level. It has been extensively used because of it has high discriminatory power for the identification of various microbial pathogens (4, 16-19). In addition, MLST technique has many advantages compared to other marker technologies such as randomly amplified polymorphic DNA (13), restriction fragment length polymorphism (20), southern blot hybridization with discriminating probes (21), amplified fragment length polymorphism analysis (22) and microsatellites (14). The genotypes determined by this technique can be compared with stored data in a central database (http://calbicans.mlst.net) (16-18, 23-25).

As regards MLST method directly analyze the polymorphism within DNA sequences; we performed the first nationwide study into the genotypic relationships of *C. albicans* strains obtained from oropharynx and bronchoalveolar lavage (BAL) samples from immunocompromised patients.

Materials and Methods

Fungal strains

During May 2006 to August 2012; 14 clinical isolates of *C. albicans* recovered from cancer patients with oropharynx lesions (n=7) and BAL samples (n=7) from Mazandaran University of Medical sciences, Sari, Iran. Stock cultures for transient working collections were initially grown on Malt extract agar (MEA) (Difco, U.S.A) at 24°C for 2-3 days. All organisms were identified to the species level by sequencing of the internal transcribed spacer (ITS) region of the rDNA (26).

MLST Analysis

MLST was used for typing the fourteen clinical isolates as previously described (18). Briefly, genomic DNA was extracted using previously described glass bead/phenol/chloroform method (27). DNA extracts were stored at -20 °C prior to use. MLST was performed based on the seven housekeeping genes, i.e., *AAT1a*, *ACC1*, *ADP1*, *MPIb*, *SYA1*, *VPS13*, and *ZWF1b* (18). The primers used for amplification and sequencing are shown in Table 1.

Table 1: Gene fragments and primers used for MLST analysis

Fragment	Gene product	Primer sequence	Amplicon size (bp)
*AAT1*a	Aspartate aminotransferase	Fwd 5'-ACTCAAGCTAGATTTTTGGC-3' Rev 5'-CAGCAACATGATTAGCCC-3'	478
ACC1	Acetyl-coenzyme A carboxylase	Fwd 5'-GCAAGAGAAATTTTAATTCAATG-3' Rev 5'-TTCATCAACATCATCCAAGTG-3'	519
ADP1	ATP-dependent permease	Fwd 5'-GAGCCAAGTATGAATGATTTG-3' Rev 5'-TTGATCAACAAACCCGATAAT-3'	537
MPI b	Mannose phosphate isomerase	Fwd 5'-ACCAGAAATGGCCATTGC-3' Rev 5'-GCAGCCATGCATTCAATTAT-3'	486
SYA1	Alanyl-RNA synthetase	Fwd 5'-AGAAGAATTGTTGCTGTTACTG-3' Rev 5'-GTTACCTTTACCACCAGCTTT-3'	543
VPS13	Vacuolar protein sorting protein	Fwd 5'-TCGTTGAGAGATATTCGACTT-3' Rev 5'-ACGGATGGATCTCCAGTCC-3'	741
*ZWF1*b	Glucose-6-phosphate dehydrogenase	Fwd 5'-GTTTCATTTGATCCTGAAGC-3' Rev 5'-GCCATTGATAAGTACCTGGAT-3'	702

PCR reactions were performed on a thermal cycler (Biorad-C1000) in a 25 µl volumes containing 25 ng of template DNA, 2.5 µl reaction buffer (0.1 M Tris-HCl, pH 8.0, 0.5 M KCl, 15 mM MgCl2, 0.1% gelatine, 1% Triton X-100), 0.1 mM of each dNTP and 1.0 U Taq DNA polymerase (ITK Diagnostics, Leiden, The Netherlands). Amplification of those genes were performed with cycles of 5 min at 94°C for primary denaturation, followed by 35 cycles at 94 °C (30s), 55 °C (60 s) and 72 °C (60 s), with a final 5 min extension step at 72 °C. Amplicons were purified using QIAquick DNA and gel band purification kit (Cat. No 28104, U.K).

Sequencing was done on an ABI 3730XL automatic sequencer (Applied Biosystems, Foster City, CA, U.S.A). Alignments of sequence data were adjusted manually for each gene using MEGA 5.05 (http://www.megasoftwa-re.net/) and Boiedit ver. 7.0.9 (http://www.mb-io.ncsu.edu/BioEdit/bioedit.html) softwares. The two overlapping strands were aligned and analyzed peak-by-peak. The heterozygosity was identified by the presence of two peaks at the same polymorphic loci on both strands and the consensus sequences of seven loci of all isolates were determined. 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). The chromatograms of new alleles and DSTs were assigned to central MLST database curator. The eBURST analysis (http://eburst.mlst.net/) was performed and DSTs of 14 isolates were compared with all available DSTs (n = 2099) in the database at July 2014 and split into eBURST clonal clusters. The UPGMA dendrogram based on MLST sequence data was drawn using CLC Sequence Viewer 6 software (http://www.clcbio.com/) which can analyze the heterozygous code data for nucleotide *P*-distances.

Results

Fourteen *C. albicans* specimens from immunocompromised patients were used in this study and all specimens were successfully typed by evaluating the DNA sequences of the fragments from seven different housekeeping genes, which yielded a set of 2,883 nucleotides for each isolate. MLST analysis showed that seventy-one (2.5%) nucleotide sites found to be variable and all of the isolates were found to be heterozygous. Sixty different alleles were identified in seven loci of the fourteen *C. albicans* isolates; *VPS13* generated the most number of alleles (n = 12), while *ADP1* generated the least (n = 7) (Table2).

Table 2: Characteristics of the seven housekeeping loci studied

Locus	Sequenced fragment size (bp)	Polymorphic sites	No. of variable bases	No. of alleles
*AAT1*a	373	7,28,40,70,89,124,325,352,361,373	10	8
ACC1	407	8,29,83,179,281,392	6	8
ADP1	443	17,40,46,109,125,166,205,215,225,232	10	7
MPI b	375	21,27,34,36,66,72,88,94,107,234,237,276,289	13	8
SYA1	391	1,25,61,100,142,160,307,351	8	9
VPS13	403	49,134,212.217,241,281,282,320,322,326,334,370,375	13	12
*ZWF1*b	491	23,31,43,49,55,175,262,274,337,379,482	11	8

Among sixty alleles, two new alleles were determined in *ADP1* locus (Allelic number 123) and other was in *MPIb* locus (Allelic number 130) and added to the MLST database (http://calbicans.mlst.net). As shown in Table 2, the sequenced genes yielded a total of 71 variable sites which *VPS13* and *MPIb* loci produced the highest (n = 13) of polymorphic sites, while *ACC1* produced the lowest number (n=6). The allelic diversity at the seven loci for the 14 *C. albicans* isolates has been found in twelve unique DSTs, which all DSTs submitted into www.calbicans.mlst.net. Among these DSTs, seven

DSTs belonged to previously described DSTs, and the five novel DSTs added to the database. The ID numbers of five new STs, which are assigned in www.calbicans.mlst.net, are 2246, 2248 and 2252 up to 2254. The novel DSTs are available in the *C. albicans* MLST database. The eBURST package (http://eburst.mlst.net/) was used to analyze the genotype relationships comparing with those available DSTs (n = 2099) in MLST database at July 2014. A total 108 eBURST clusters and 707 singleton strains produced. Twelve isolates were placed in 10 clonal clusters (CC) while two isolates were singletons.

Results revealed 10 out of 14 strains had a different DSTs, but two isolates; AF24 and AF52; had identical DSTs 172 which belonged to CC 172 and also two other isolates; AF57 and AF60; had same DST 1751 which assigned to CC 461 (Fig. 1). One the isolates with new DST 1110 is clustered in CC 344 and identified as ST bootstrap subgroup 99% (Fig. 2). As shown in Fig. 2, DST 1110 is subset of CC 344 and known as ST bootstrap of 35 strains. The UPGMA dendrogram for the concatenated variable sequences from the 14 isolates with different source clearly showed the clustering pattern (Fig.1).

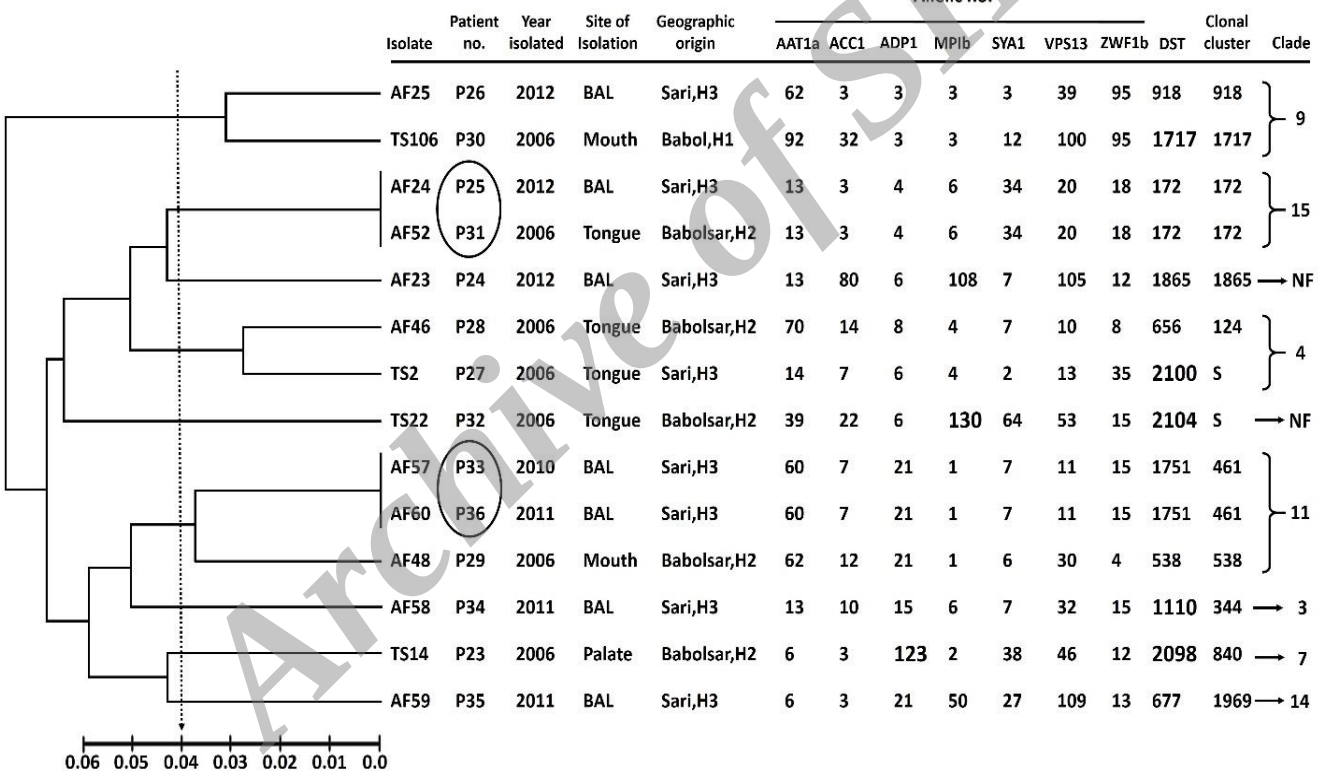

Fig. 1: Summarizes the all information of 14 *C. albicans* clinical isolates analyzed by MLST technique. This comprises the allelic number for each locus, DSTs, clonal clusters, clades and it also illustrates the UPGMA dendrogram based on MLST data. All geographic origins were situated in Mazandaran, Iran
H1: Amir kola Hospital; H2: Rajaee Hospital; H3: Imam Khomeini Hospital.
Bold numbers are new alleles and DSTs.
S: Singleton
NF: Not found
*: Same DSTs from different patients

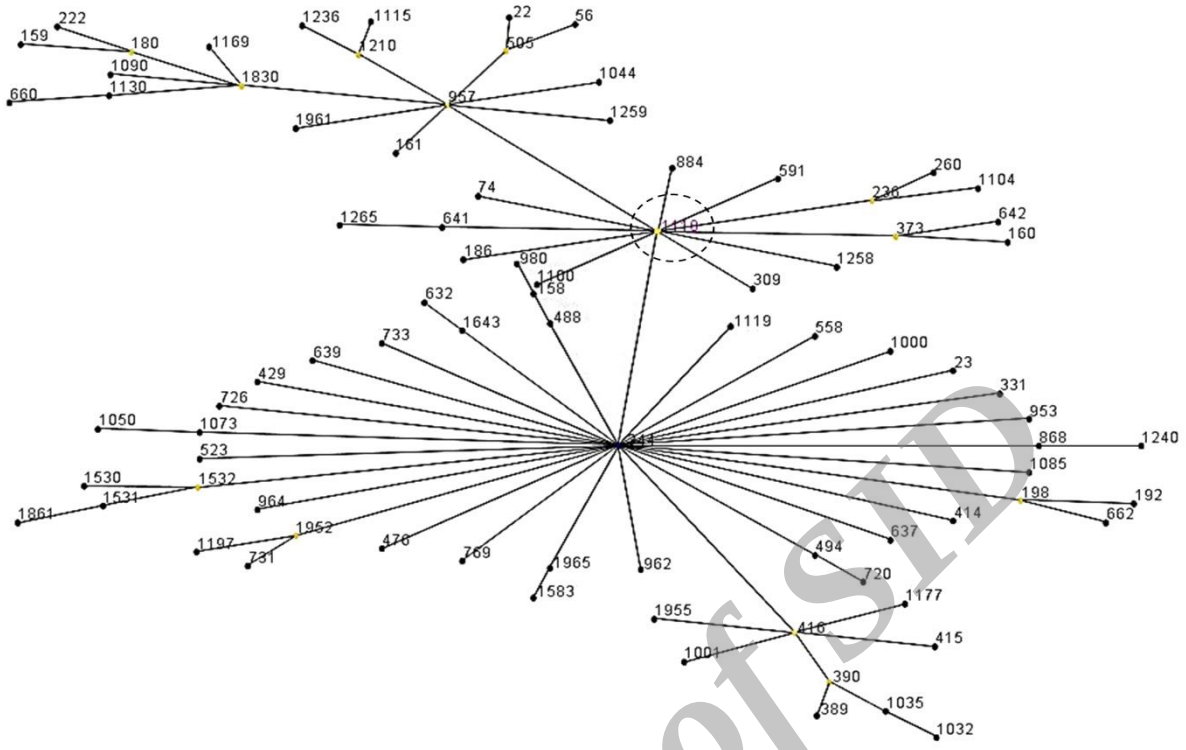

Fig. 2: New DST 1110 from BAL sample is clustered in CC 344 by eBURST analysis and identified as ST bootstrap subgroup 99%

The MLST results have showed that some isolates have the same DST. In total among 14 unrelated clinical isolates (oropharynx and BAL samples of immunocompromised patients), showed 12 different DSTs (85.7%). The same DST 1751 profile was found among two patients; P33 and P36; and same DST 172 discovered in two patients; P18 and P31.

Discussion

Strain typing, or the ability to distinguish between clonally unrelated isolates, is an indispensable tool in the clinical microbiology laboratory and for hospital epidemiology. Some of the most important reasons to perform strain typing in the mycology field are patient monitoring and treatment follow-up, outbreak analysis, environmental monitoring and the study of local and global epidemiology of fungal pathogens.

MLST is one of a series of techniques for typing studies and it is highly discriminative method that using in this context can detect micro variations and microevolutions in the genomes of epidemiological related strains of *C. albicans* (9, 17, 28). A discriminatory strain typing system would be useful for clinical and epidemiological studies to obtain information of sources, carriage, transmission, correlation and properties, i.e., virulence and antimicrobial resistance (14, 29). This method is also used to the study of *C. albicans* isolates obtained from different patients at different times and locations, which are known as epidemiologically unrelated strains and usually are not genetically closely related and classify in different clusters (17, 23).

In the present study, we investigated the genetic diversity of *C. albicans* strains and now discuss the implications of a comparison of 14 clinical isolates. Based on the MLST of seven loci we classified strains of *C. albicans* into seven clades, which were supported by high bootstrap values (Fig. 1).The aim of this study was to evaluate the genetic diversity and relationships among unrelated *C. albicans* isolates recovered from oropharynx and BAL samples. We used MLST to determine *C. albicans*

genotypes, and found that ten patients (71.5%) were infected by ten strains with different DST. Two strains with identical MLST patterns were isolated from two patients; P33 and P36; who admitted at the same infectious disease ward in different time. Therefore, it seems that colonized patients are a main reservoir of *C. albicans* in hospitals and the isolation of *C. albicans* with the same DST in more than one patient is remarkable and may provide evidence for cross contamination between patients, which might be a crucial factor for nosocomial infections. In addition, two other strains with the same DST were isolated from two patients; P18 and P31; admitted in different hospital. Sum total 12 unrelated isolates belonged to seven out of 17 clades, (4, 19), while 2 isolates not assigned into defined clades. Odds showed that the five clades (numbered 1,2,3,4 and 11) are the largest clades at MLST database of *C. albicans* (19) and among 14 isolates of this study, six strains (43%) belonged to three out of this five clades (3, 4, 11). None of our isolates belonged to clades 1 and 2. The five (36%) of our isolates were new DST which one of those (DST 1110) was clustered in CC 344 based on eBURST analysis and identified as ST bootstrap and 35 strains were located as subset of DST 1110. In other our study, Afsarian and colleagues applied MLST for 30 epidemiologically related *C. albicans* isolates and acquired 18 DSTs and was identified significant genetic relation among the epidemiologically related *C. albicans* isolates from burn patients at the same burn ICU during the same 6 months (30). Tavanti and colleagues analyzed 416 unrelated *C. albicans* isolates by the eBURST software and 228 (54.8%) were assigned to clonal clusters which belonged to 12 clades. Almost half of the isolates were left as singleton (23). Bougnoux and co-workers found 27 DSTs among the 28 epidemiologically unrelated *C. albicans* isolates. They indicated that only two isolates had identical DSTs. Therefore, they showed MLST of 14 epidemiologically related isolates that recovered from patients in the same hospital ward during the same 3 months had specific DSTs, although 73% of these isolates were genetically related (17). Therefore, according to the previous studies (17, 23, 30) and obtained data in current study indicate that genetic diversity is variable among unrelated strains isolated from different patients and from non-identical places. Therefore, MLST generates evidence for similarities and differences between isolates from sequences determined and MLST schemes provide a high discriminatory power when applied to a collection of epidemiologically unrelated isolates.

Conclusion

Isolates that show identical or very similar allelic profiles are presumed identical or closely related. This would be used to evaluate the relationships between isolates identified within a specific environment. Pathogen distribution and relatedness for determining the epidemiology of nosocomial infections is highly recommended for pathogen control methods.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

Acknowledgments

This research was supported by Mazandaran University of Medical Sciences, Sari, Iran (grant number 91-32) which the authors gratefully acknowledge. The authors acknowledge the use of the *Candida albicans* MLST database, located at Imperial College London and is funded by the Welcomes Trust. We also thank the curator of MLST database, Marie-Elisabeth Bougnoux for assigning our new alleles and STs to *C. albicans* MLST database. We would also like to show our gratitude to Iman Haghani and Sabah Myahi for technical assistance.
The authors declare that there is no conflict of interests.

References

1. Calderone RA, Clancy CJ (2011). *Candida and candidiasis*. 2nd ed. Calderone and Clancy ASM Press, USA, pp.:1-13.
2. Odds FC (1988). *Candida and candidosis: a review and bibliography*. 2nd ed. Bailliere Tindall, London, pp.:1-17.
3. Pfaller M, Diekema D (2007). Epidemiology of invasive candidiasis: a persistent public health problem. *Clin Microbiol Rev,* 20(1):133-63.
4. Odds FC, Bougnoux M-E, Shaw DJ, Bain JM, Davidson AD, Diogo D, Jacobsen MD, Lecomte M, Li S-Y, Tavanti A (2007). Molecular phylogenetics of *Candida albicans*. *Eukaryot cell,* 6(6):1041-52.
5. Bougnoux M-E, Diogo D, Francois N, Sendid B, Veirmeire S, Colombel J, Bouchier C, Van Kruiningen H, d'Enfert C, Poulain D (2006). Multilocus sequence typing reveals intrafamilial transmission and microevolutions of *Candida albicans* isolates from the human digestive tract. *J Clin Microbiol,* 44(5):1810-20.
6. Horn DL, Neofytos D, Anaissie EJ, Fishman JA, Steinbach WJ, Olyaei AJ, Marr KA, Pfaller MA, Chang C-H, Webster KM (2009). Epidemiology and outcomes of candidemia in 2019 patients: data from the prospective antifungal therapy alliance registry. *Clin Infect Dis,* 48(12):1695-703.
7. Shokohi T, Bandalizadeh Z, Hedayati MT, Mayahi S (2011). In vitro antifungal susceptibility of *Candida* species isolated from oropharyngeal lesions of patients with cancer to some antifungal agents. *Jundishapur J Microbiol,* 4(1): 19-26.
8. Eggimann P, Garbino J, Pittet D (2003). Epidemiology of *Candida* species infections in critically ill non-immunosuppressed patients. *Lancet Infect Dis,* 3(11):685-702.
9. Bougnoux M-E, Kac G, Aegerter P, d'Enfert C, Fagon J-Y (2008). Candidemia and candiduria in critically ill patients admitted to intensive care units in France: incidence, molecular diversity, management and outcome. *Intensive Care Med,* 34(2):292-9.
10. Reagan DR, Pfaller MA, Hollis RJ, Wenzel RP (1995). Evidence of nosocomial spread of *Candida albicans* causing bloodstream infection in a neonatal intensive care unit. *Diagn Microbiol Infect Dis,* 21(4):191-4.
11. Schmid J, Tay YP, Wan L, Carr M, Parr D, McKinney W (1995). Evidence for nosocomial transmission of *Candida albicans* obtained by Ca3 fingerprinting. *J Clin Microbiol,* 33(5):1223-30.
12. Marco F, Lockhart S, Pfaller M, Pujol C, Rangel-Frausto M, Wiblin T, Blumberg H, Edwards J, Jarvis W, Saiman L (1999). Elucidating the origins of nosocomial infections with *Candida albicans* by DNA fingerprinting with the complex probe Ca3. *J Clin Microbiol,* 37(9):2817-28.
13. Robert F, Lebreton F, Bougnoux M, Paugam A, Wassermann D, Schlotterer M, Tourte-Schaefer C, Dupouy-Camet J (1995). Use of random amplified polymorphic DNA as a typing method for *Candida albicans* in epidemiological surveillance of a burn unit. *J Clin Microbiol,* 33(9):2366-71.
14. Garcia-Hermoso D, Cabaret O, Lecellier G, Desnos-Ollivier M, Hoinard D, Raoux D, Costa J-M, Dromer F, Bretagne S (2007). Comparison of microsatellite length polymorphism and multilocus sequence typing for DNA-based typing of *Candida albicans*. *J Clin Microbiol,* 45(12):3958-63.
15. Mirhendi S, Makimura K (2003). PCR-detection of *Candida albicans* in blood using a new primer pair to diagnosis of systemic candidiasis. *Iran J Public Health,* 32(1):1-5.
16. Bougnoux M-E, Aanensen DM, Morand S, Théraud M, Spratt BG, d'Enfert C (2004). Multilocus sequence typing of *Candida albicans*: strategies, data exchange and applications. *Infect Genet Evol,* 4(3):243-252.
17. Bougnoux M-E, Morand S, d'Enfert C (2002). Usefulness of multilocus sequence typing for characterization of clinical isolates of *Candida albicans*. *J Clin Microbiol,* 40(4):1290-7.
18. Bougnoux M-E, Tavanti A, Bouchier C, Gow N, Magnier A, Davidson A, Maiden M, d'Enfert C, Odds F (2003). Collaborative consensus for optimized multilocus sequence typing of *Candida albicans*. *J Clin Microbiol,* 41(11):5265-6.
19. Odds FC (2010). Molecular phylogenetics and epidemiology of *Candida albicans*. *Future Microbiol,* 5(1):67-79.
20. Shokohi T, Hashemi Soteh M, Pouri ZS, Hedayati M, Mayahi S (2010). Identification of *Candida* species using PCR-RFLP in cancer

patients in Iran. *Indian J Med Microbiol,* 28(2):147-51.

21. Myoung Y, Shin JH, Lee JS, Kim SH, Shin MG, Suh SP, Ryang DW (2011). Multilocus sequence typing for Candida *albicans* isolates from candidemic patients: comparison with Southern blot hybridization and pulsed-field gel electrophoresis analysis. *Korean J Lab Med,* 31(2):107-14.
22. Borst A, Theelen B, Reinders E, Boekhout T, Fluit A, Savelkoul P (2003). Use of amplified fragment length polymorphism analysis to identify medically important *Candida* spp., including C. dubliniensis. *J Clin Microbiol,* 41(4):1357-62.
23. Tavanti A, Davidson AD, Fordyce MJ, Gow NA, Maiden MC, Odds FC (2005). Population structure and properties of *Candida albicans*, as determined by multilocus sequence typing. *J Clin Microbiol,* 43(11):5601-13.
24. Tavanti A, Gow NA, Senesi S, Maiden MC, Odds FC (2003). Optimization and validation of multilocus sequence typing for *Candida albicans*. *J Clin Microbiol,* 41(8):3765-76.
25. Shin JH, Bougnoux M-E, d'Enfert C, Kim SH, Moon C-J, Joo MY, Lee K, Kim M-N, Lee HS, Shin MG (2011). Genetic diversity among Korean *Candida albicans* bloodstream isolates: assessment by multilocus sequence typing and restriction endonuclease analysis of genomic DNA by use of BssHII. *J Clin Microbiol,* 49(7):2572-7.
26. Mohammadi R, Mirhendi H, Rezaei-Matehkolaei A, Ghahri M, Shidfar MR, Jalalizand N, Makimura K (2013). Molecular identification and distribution profile of *Candida* species isolated from Iranian patients. *Med Mycol,* 51(6):657-63.
27. Yamada Y, Makimura K, Merhendi H, Ueda K, Nishiyama Y, Yamaguchi H, Osumi M (2002). Comparison of different methods for extraction of mitochondrial DNA from human pathogenic yeasts. *Jpn J Infect Dis,* 55(4):122-5.
28. Maiden MC, Bygraves JA, Feil E, Morelli G, Russell JE, Urwin R, Zhang Q, Zhou J, Zurth K, Caugant DA (1998). Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc Natl Acad Sci U S A,* 95(6):3140-5.
29. Odds FC, Jacobsen MD (2008). Multilocus sequence typing of pathogenic *Candida* species. *Eukaryot cell,* 7(7):1075-84.
30. Afsarian MH, Badali H, Boekhout T, Shokohi T, Katiraee F (2015). Multilocus sequence typing of *Candida albicans* isolates from a burn intensive care unit in Iran. *J Med Microbiol*, 64(3):248-53.