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# Disparity between Multilocus Enzyme Electrophoresis, Microsatellite Markers and Pulsed-Field Gel Electrophoresis in epidemiological tracking of *Candida albicans*

Marcelo Fabiano Gomes Boriollo [a,*], Ricardo Antunes Dias [a], João Evangelista Fiorini [b], Nelma de Mello Silva Oliveira [b], Denise Madalena Palomari Spolidório [c], Henrique Marques Barbosa de Souza [d], Antonio Vargas de Oliveira Figueira [d], Aline Aparecida Pizzirani-Kleiner [e]

[a] *Laboratory of Molecular Biology and Genetics, Faculty of Medical Sciences, University of Alfenas, Minas Gerais, Brazil*
[b] *Laboratory of Biology and Physiology of Microorganisms, Faculty of Medical Sciences, University of Alfenas, Minas Gerais, Brazil*
[c] *Laboratory of Oral Microbiology, Department of Oral Pathology and Physiology, Faculty of Ordontology of Araraquara, State University of São Paulo, Araraquara, São Paulo, Brazil*
[d] *Laboratory of Plant Improvement, Center of Nuclear Energy in Agriculture, University of São Paulo, Piracicaba, SP, Brazil*
[e] *Department of Genetics, Escola Superior de Agricultura Luiz de Queiroz, University of São Paulo, Piracicaba, SP, Brazil*

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ABSTRACT

Various molecular systems are available for epidemiological, genetic, evolutionary, taxonomic and systematic studies of innumerable fungal infections, especially those caused by the opportunistic pathogen *C. albicans*. A total of 75 independent oral isolates were selected in order to compare Multilocus Enzyme Electrophoresis (MLEE), Electrophoretic Karyotyping (EK) and Microsatellite Markers (Simple Sequence Repeats - SSRs), in their abilities to differentiate and group *C. albicans* isolates (discriminatory power), and also, to evaluate the concordance and similarity of the groups of strains determined by cluster analysis for each fingerprinting method. Isoenzyme typing was performed using eleven enzyme systems: *Adh*, *Sdh*, *M1p*, *Mdh*, *Idh*, *Gdh*, *G6pdh*, *Asd*, *Cat*, *Po*, and *Lap* (data previously published). The EK method consisted of chromosomal DNA separation by pulsed-field gel electrophoresis using a CHEF system. The microsatellite markers were investigated by PCR using three polymorphic loci: *EF3*, *CDC3*, and *HIS3*. Dendrograms were generated by the SAHN method and UPGMA algorithm based on similarity matrices ($S_{SM}$). The discriminatory power of the three methods was over 95%, however a paired analysis among them showed a parity of 19.7-22.4% in the identification of strains. Weak correlation was also observed among the genetic similarity matrices ($S_{SM}{}^{MLEE} \times S_{SM}{}^{EK} \times S_{SM}{}^{SSRs}$). Clustering analyses showed a mean of $9 \pm 12.4$ isolates per cluster ($3.8 \pm 8$ isolates/*taxon*) for MLEE, $6.2 \pm 4.9$ isolates per cluster ($4 \pm 4.5$ isolates/*taxon*) for SSRs, and $4.1 \pm 2.3$ isolates per cluster ($2.6 \pm 2.3$ isolates/*taxon*) for EK. A total of 45 (13%), 39 (11.2%), 5 (1.4%) and 3 (0.9%) clusters pairs from 347 showed similarity ($S_J$) of 0.1-10%, 10.1-20%, 20.1-30% and 30.1-40%, respectively. Clinical and molecular epidemiological correlation involving the opportunistic pathogen *C. albicans* may be attributed dependently of each method of genotyping (i.e., MLEE, EK, and SSRs) supplemented with similarity and grouping analysis. Therefore, the use of genotyping systems that give results which offer minimum disparity, or the combination of the results of these systems, can provide greater security and consistency in the determination of strains and their genetic relationships.



## 1. Introduction

*Candida albicans* and related species are found ubiquitously and commensally in the cavities (rectal, oral, vaginal, urethral, nasal, and aural) and skin microbiota of humans (Segal and Baum, 1994). These species are considered opportunistic pathogens capable of causing infections varying from mucocutaneous disorders, not compromising to the individual, to invasive diseases involving almost all organs. The frequency of infections caused by *Candida* spp. has been increasing worldwide due to a multiplicity of predisposing factors that facilitate its conversion from a commensal to a parasitic form (Akpan and Morgan, 2002; Samaranayake, 1990; Samaranayake et al., 2001). The revolution in molecular biology has dramatically increased the ability of researchers to study innumerable fungal infections, especially those caused by the opportunistic pathogen *C. albicans* and other *Candida* species. Various molecular systems such as MLEE (*Multilocus Enzyme Electrophoresis*), EK (*Electrophoretic Karyotyping*), RAPD (*Randomly*

* Corresponding author. Laboratory of Molecular Biology and Genetics, Medical Sciences School, University of Alfenas, Rod. MG 179, Km 0 - Universitary Campus, CEP 37130-000, Alfenas, MG, Brazil. Tel.: +55 35 3299 3255.
*E-mail address:* marcelo.boriollo@unifenas.br (M.F.G. Boriollo).

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*Amplified Polymorphic* DNA), RFLP without hybridization (*Restriction Length Fragment Polymorphism without hybridization*) or REA (*Restriction Enzyme Analysis*), RFLP with hybridization (*Restriction Length Fragment Polymorphism with hybridization*), and Microsatellite Markers or SSRs (*Simple Sequence Repeats)* are available for epidemiological, genetic, evolutionary, taxonomic and systematic studies.

Pioneering work in the 1980 s, employing MLEE for the genetic analysis of *Escherichia coli* and *Shigella*, created enormous interest among medical microbiologists (Selander and Lewin, 1980; Selander et al., 1986). Henceforth, numerous studies were conducted producing immeasurable findings toward the understanding of the natural history of infectious diseases. In the field of medical mycology, isoenzyme typing has shown great potential in studies of taxonomic, systematic, genetic, evolutive, and epidemiologic characterization, especially for the yeast *C. albicans* (Arnavielhe et al., 1996, 1997; Barchiesi et al., 1998; Boerlin et al., 1996; Boriollo et al., 2005, 2006; Pujol et al., 1993a, 1993b, 1997; Rosa et al., 1999, 2000a, 2000b, 2001, 2003). MLEE has been considered the gold standard in the study of population genetics of microorganisms (Boerlin, 1997). Its analytical capacity allows the study of codominant markers for each locus in diploid organisms, an essential requirement for evolutionary biologists, which is not achieved by some of the popular DNA fingerprinting methods (Soll, 2000). Species-specific clones identified by MLEE were also associated with clinical patterns and re-incidence, and high indices of pathogenicity. Such results have been employed in epidemiologic delineations permitting better understanding of the development of epidemics, in which it represents an immeasurable complement to current methods of molecular typing, particularly in large-scale epidemiologic studies. Additionally, MLEE has been considered a typing method with high discriminatory power and reproducibility (Boerlin, 1997; Boerlin et al., 1996; Hunter, 1991; Murphy et al., 1990; Pasteur et al., 1987; Pujol et al., 1997; Boriollo et al., 2006).

Simple Sequence Repeats (SSRs) or microsatellites are tandemly repeated motifs of 1 to 6 bases found in all prokaryotic and eukaryotic genomes. They are present in both coding and noncoding regions (Falconer and Mackay, 1996; Hartl and Clark, 1997; Van Belkum, 1999; Zane et al., 2002) and are usually characterized by a high degree of length polymorphism. The origin of such genetic variability is still under debate though it most likely to be due to slippage events during DNA replication (Schlötterer and Tautz, 1992), often in combination with defective DNA mismatch repair (Strand et al., 1993). Consequently, these variations can alter the genetic repertoire of a particular (micro)organism and can result in evolution of the species (Van Belkum, 1999). Although the microsatellite evolutionary mechanism still remains uncertain, SSRs have been considered efficient genetic markers because of their high variability. The high reproducibility and stability of microsatellite markers, easily demonstrated by automated PCR and electrophoresis procedures (Gotttfredsson et al., 1998; Van Belkum et al., 1998; Zane et al., 2002), have been reported by various researchers in their epidemiological studies involving the opportunistic pathogen *C. albicans.* However, only a few polymorphic microsatellite *loci* have been identified in the genome of *C. albicans*, with most located next to or within coding regions and exhibiting a high discriminatory power (Botterel et al., 2001; Bretagne et al., 1997; Costa et al., 2005; Field et al., 1996; Fundyga et al. 2002; Garcia-Hermoso et al., 2007; Lockhart et al., 1995; Lott et al., 2003; Lunel et al., 1998; Metzgar et al., 1998a, 1998b; Rohatiner, 1996; Sampaio et al., 2003, 2005; Stéphan et al., 2002). However, the greatest degree of polymorphism in microsatellite *loci* was observed in non-coding regions and few studies have been developed for analysis of these regions in *C. albicans* (Lott and Effat, 2001; Lunel et al., 1998; Sampaio et al., 2003).

Pulsed-field gel electrophoresis (PFGE) (Schwartz and Cantor, 1984), and its respective systems OFAGE (*orthogonal-field alternative gel electrophoresis*), FIGE (*field inversion gel electrophoresis*), CHEF (*contour clamped homogeneous electric field*) or TAFE (*transverse alternate field electrophoresis*), permit easy separation of chromosomal fragments from the genome of yeast in a gel, thereby consolidating Electrophoretic Karyotyping (Soll, 2000). The first applications of PFGE with unrelated isolates of *C. albicans* showed variations in their karyotypic patterns (Kwon-Chung et al., 1988; Lasker et al., 1989; Lott et al., 1987; Magee and Magee, 1987; Merz et al., 1988; Snell and Wilkins, 1986; Suzuki et al., 1988). Their electrophoretic patterns were considered highly reproducible between experiments, relatively intensive in the preparation of methods within the same laboratory, and not influenced by high-frequency phenotypic switching in strains of *C. albicans* (Sangeorzan et al., 1995). Due to its excellent discriminatory power and reproducibility (Bostock et al., 1993; Gottfredsson et al., 1998; Magee et al., 1992; Reiss et al., 1998; Vazquez et al., 1991), electrophoretic karyotyping has been employed extensively as a molecular marker for *C. albicans* (Barchiesi et al., 1995; Bart-Delabesse et al., 1995; Doi et al., 1994; Espinel-Ingroff et al., 1996; Lasker et al., 1989; Lott et al., 1987; Lupetti et al., 1995; Magee et al., 1987, 1992; Merz et al., 1988; Pfaller et al., 1994; Snell and Wilkins, 1986; Suzuki et al., 1988; Vazquez et al., 1991). However, its efficiency in grouping moderately related strains has not been carefully evaluated (Soll, 2000) and also the high cost and relatively long time required to carry out the assay, which consequently reduces the laboratory capacity to analyze large numbers of samples, have limited its use (Olive and Bean, 1999; Reiss et al., 1998).

Based on data from available literature, the objective of this study was to determine the discriminatory power and similarity of the Multilocus Enzyme Electrophoresis (published in Boriollo et al., 2006), Microsatellite Markers and Electrophoretic Karyotyping methods of molecular typing from their respective genetic (deduction of allelic composition of diploid organisms) and numerical interpretations (simple count of the presence and absence of bands) of the electrophoretic patterns and consequent identification of strains. Then, to determine the parity or disparity between groups of identical and highly related isolates obtained by similarity and grouping analysis commonly used in epidemiological investigation and molecular typing of fungal infections.

## 2. Material and methods

### 2.1. Yeast isolates

The study involved 75 specimens of *C. albicans* isolated from the oral cavities of 75 clinically healthy children (one isolate per child), characterized previously by the group of investigators at the Laboratory of Microbiology and Immunology of the Department of Oral Diagnostics, Dental School of Piracicaba, State University of Campinas (Moreira et al., 2001; Boriollo et al., 2005).

### 2.2. Multilocus Enzyme Electrophoresis (MLEE)

Enzyme extraction, electrophoresis and specific enzyme staining, and interpretation of MLEE patterns (numeric and genetic) were previously published (Boriollo et al., 2006). MLEE was found to be a powerful and reliable tool for the typing of *C. albicans* due to its high discriminatory power (>0.9). However, disparities were observed during clusters analyses based on UPGMA dendrograms and elements of two matrices (similarity and/or genetic distance).

### 2.3. Microsatellite Markers – Simple Sequence Repeats (SSRs)

DNA extraction of *C. albicans* oral isolates grown freshly on YEPD medium was accomplished using a slightly modified, previously described protocol (Piper, 1996). The DNA solutions (TE buffer) were stored at -20 °C and their quantification was accomplished by spectrophotometry at 260 nm (Genesys™ 10 Series Spectrophotometers, Spectronic Unicam, New York, USA). Genotypes of three