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CANDIDA ALBICANS: GENOTYPING METHODS AND CLADE RELATED PHENOTYPIC CHARACTERISTICS

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ABSTRACT

Several molecular methods, such as Southern blotting hybridization, Multilocus Sequence Typing, and DNA microsatellite analysis, have been employed to genotype Candida albicans. The genotype analysis allows to group strains in clades, that is, a group composed of one ancestor and its descendants. These genotype studies demonstrate that clades distribution is influenced by geographic area as well as that antifungal resistance is associated with particular clades. These findings suggested that *C. albicans* reproduces mainly in a clonal manner, with certain degree of DNA microevolution. Additionally, virulence factors and site of isolation have also been associated with clade specificity. The present article is a brief review about the methods used for *Candida* genotyping and the correlated clade systems established. Special emphasis is given to Ca3 hybridization, MLST, and Microsatellites. The present work is also focused on the phenotypic and physiological traits associated with *Candida* clades.

Key words: *Candida albicans*; clonal reproduction; microevolution; genotyping systems; phenotypic characteristics.

INTRODUCTION

Candida yeasts components of normal human microbiota, being present in a large percentage of healthy individuals (36). However, in a diverse range of debilitated and immunocompromised hosts, *Candida* infections may cause relevant number of morbidity and mortality cases which constitute a serious public health problem (21, 23). Conditions such as immunodeficiency, endocrine disorders, radiotherapy or malignant diseases can be identified as predisposing factors to candidiasis. Several species have been reported to be responsible for infection such as *C. krusei*, *C. tropicalis*, *C. glabrata*, and *C. guilliermondii*; however, *C. albicans* has been considered the major etiological agent between them (11, 15, 22, 31).

Several methodologies have been used for typing *C. albicans*, such as electrophoretic karyotyping (14, 16, 43, 52) Restriction Fragment Length Polymorphism (RFLP) (12), Random Amplified Polymorphic DNA (RAPD) (5, 24, 36), Multilocus Enzyme Electrophoresis (MLEE) (2, 6, 45), Microsatellites (7, 28), Multilocus Sequence Typing (MLST) (9, 32, 58), and DNA fingerprinting with Ca3 probe (4, 40). The present review focus on DNA probe Ca3, MLST, and Microsatellites. The experiments developed with these techniques can be reproduced (38), results obtained from different laboratories can be compared with a high degree of

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confidence (49). The techniques mentioned are employed for the classification of *Candida* strains into groups formed by a single ancestor and its descendants, which are also called clades.

C. albicans is a diploid specie with substantial heterozygosity (37), but predominant reproduces in a clonal mode (27, 30, 42). The genomic microvariation that has been documented for multiple *C. albicans* isolates from single patients (8, 27, 39, 47) occurs due to recombination (57), mitotic crossing over (37), and ploidy changes based on chromosome morphological alterations (48, 59).Odds *et al*., (33) suggested that the population of cells colonizing or infecting a particular site in a patient comprises a mixture of cells with nearly identical genomes.

The identification of microvariation between strains helps to understand the dynamics of the genetic microevolution in *Candida* population, and its connection with the appearance of drug-resistant strains. Moreover, *C. albicans* and related species undergo spontaneous high-frequency switching among a limited number of general phenotypes that reflect an immediate environmental influence such as sensitivity to antifungal agent, assimilation of carbohydrates, and even morphology alterations of the colony (54). These immediate changes in *Candida* species reflect alterations in the pattern of gene expression, required for the adjustment of their metabolic reactions needed for the maintenance of their cellular homeostasis. Thus, modern methodological techniques have been used in order to discriminate the strains in a systemic fungal infection, which is crucial for early and specific initiation of antifungal therapy.

Considerable conserved extensions of DNA are present between *C. albicans* strains, because in these organisms, microevolution works as a result of local evolutive mechanisms. The individuality of each strain can be detected based on specific tandem repeats sequences randomly distributed over the genome, which are highly polymorphic between the individuals. DNA fingerprinting techniques provide quantitative data that reflect the precise genetic distance between strains. In this way, the strains analyzed can be classified as identical, related or non-related.

In clinical investigation, DNA fingerprinting methods help on strain discrimination and on the answering of epidemiological questions such as: (i) understanding the dynamics of an infectious organism in a human population, (ii) comprehending the relationship between commensalism and infection, (iii) identifying the origin of an infection, and (iv) monitoring the emergence of drug-resistant strains (54). If such questions are solved, molecular genotyping can impact on the management of *Candida* infections.

A detailed view on *Candida* genotyping classification is discussed in the present review, and a final correlation between the phenotype characteristics of the strains and their association with clade groups is present.

DNA fingerprinting with C3a probe

One of the most common methodologies employed in genotyping through DNA fingerprinting is Restriction Length Polymorphism (RFLP) followed by the Southern blot technique and specific probe hybridization. The most successful hybridization probes for fungi are fragments containing repetitive genomic sequences, such as Ca3 (54). Ca3 is a moderately repetitive 11-kb *C. albicans* gene fragment which has been used as an effective DNA fingerprinting probe in several epidemiological studies.

When probed with the entire Ca3 fragment, EcoRI-digested DNA of *C. albicans* strain 3153A exhibit patterns composed of 10 to 20 bands of relatively high intensity and 4 to 6 bands of low intensity (1, 54). While the entire pattern of hybridization has been used to assess the relatedness of isolates, the subset of hypervariable bands have been used to monitor the microevolution of strains at sites of infection or carriage (51, 54). The software Dendron is employed to generate dendrograms, through computational analysis of the patterns obtained by Southern blotting hybridization. The software combines the results of image processing, gel image analysis, computation of similarity coefficients, genesis of dendrograms, and is also able to storage the data for future retrospective analyses (54). Because Dendron retains the

digitized Ca3 Southern blot hybridization pattern of every *C. albicans* strain analysed, and retrospectively compares all newly analysed strains with all previously analysed strains, a data bases for epidemiological studies have been developed. These data have been considered in the analysis of geographical distribution, transmission, and strain specialization (54).

The fingerprinting of *C. albicans* employing Southern blot hybridization with the midrepeat sequence Ca3 has proven to be reproducible and highly amenable to computer-assisted analysis (38). Pujol *et al* (38) used Ca3 fingerprinting to analyse the genetic relatedness of a small collection of *C. albicans* isolates recovered in the United States of America, and clustered the samples into three groups named I, II and III. Subsequently, Blignaut *et al* (3) analysed the genetic relatedness of *C. albicans* collected from the oral cavity of HIV positive and healthy South African individuals. This work revealed a South African specific group, named SA. This *C. albicans* group is present in 53% of isolated collected from black South Africans and it is also present in 33% of the isolated collected from white South Africans. These results clear demonstrated an interesting pattern of racial differences in host colonization. Furthermore, Pujol *et al* (40) identified the presence of a European-specific group (group E), and this group represented 26% of European *C. albicans* isolates. It is also interesting to note that when samples obtained in USA Southwest and South America were analysed from genetic relatedness, no isolates clustered into group II (40). The importance of this series of articles was the establishment of clades with specific characteristics that can be compared with future epidemiological assays.

Soll and Pujol (55) analyzed the geographic distribution of *Candida* isolates according to the clade system developed in the previous works. According to these authors, isolates belonging to clade I were consisted in the major group in North America, and were less representative in South Africa and Europe. Isolates belonging to calde II represented a relatively small population in North America, Europe and South Africa and this population was absent in South America and Southwest US. Clade III isolates corresponded to 23 and 24% of South America and North America isolates respectively, but ranged from 6 to 7 % among samples obtained in South Africa and Europe. Clade III isolates were more prevalent in Southwest USA (51%). Moreover, the authors analysed the regional distribution of the isolates belonging to group SA, which is strongly represented in South African strains but was poorly represented among the *C. albicans* strains from other regions studied. The distribution of Clade E isolates was predominant in Europe and appeared in a lower percentage rates in other geographic sites. Based on the above observations, and in the presence or absence of the intervenient retrotransposon IS1 sequence in the 25S rRNA gene, Soll and Pujol (2003) also speculated an evolutionary relationship between *C. albicans* strains. According to their analysis, a phylogenetic tree was suggested and in which the SA group diverged from the other strains in the initial of the evolutionary process; the other strains looked to be more correlated between themselves. That is, a common ancestor derived the SA clade and another branch. Clades I, II, III and E are derived from this earlier branch.

Another interesting work was developed by Edelmann *et al* (18) using DNA fingerprinting with Ca3 probe. They verified the genetic relatedness of *C. albicans* cultures obtained from human and animal sources. The phylogenetic analysis did not reveal the existence of species-specific lineages, which suggested that animals could be possible resources for *Candida* infection. However, although no species specificity has been demonstrated, different *C. albicans* clades may differ in the frequency in which they colonize various species.

DNA fingerprinting with the complex probe Ca3 thus represents an interesting method for *Candida* genotyping which has been contributing to the understanding of the differences between strains traits.

Multilocus Sequence Typing

Multilocus sequence typing is a highly discriminatory and practical approach to distinguishing strains within microorganism species (29). Molecular typing data obtained

through MLST can be shared via internet (http://www.mlst.net), allowing global epidemiologic analysis. The technique is used for typing multiple loci in the genomic DNA and became the method of choice for *Candida* typing (9, 10). MLST involves DNA amplification by Polymerase Chain Reaction (PCR) followed by DNA sequence. It measures the DNA sequence variation in a set of housekeeping genes and characterizes strains by their unique allelic profiles. The characterization is based on the analysis of nucleotide polymorphisms of the sequences of approximately 450- to 500-bp internal fragments (loci) of housekeeping genes. For each housekeeping locus, different sequences present within the species are considered as distinct alleles. The large number of alleles at each housekeeping gene analyzed permits the construction of different allelic profiles. These data can be used to construct a dendogram using the matrix of pairwise differences between their allelic profiles that can be assumed to be derived from a common ancestor.

Bougnoux *et al* (9) and Tavanti *et al* (58) were the first researches that described a set of gene fragments for MLST of *C. albicans*. Based on these previous efforts, Bougnoux *et al* (2003) developed an optimized protocol for MLST of *C. albicans*. The authors proposed the following set of gene fragments as international standard protocol for MSLT in the fungi: *AAT1a, ACC1, ADPI, MPIb*, *SYA1, VPS13,* and *ZWF1b*. This set yielded unique diploid sequence types for each isolate tested.

According to Chowdahry *et al* (13), MLST can be applied to define genetic relatedness of sequential *C. albicans* isolates, achieving equal or even better results than when the analysis is performed by Ca3 Southern hybridization. Similarly, Robles *et al* (44) demonstrated that MLST is at least comparable with random amplified polymorphic DNA (RAPD), multilocus enzyme electrophoresis (MLEE) and Southern blotting in discriminating *C. albicans* strains.

When data obtained by MLST for *C. albicans* were analysed by unweighted-pair group method with arithmetic mean (UPGMA), the major clades obtained correlate extremely well with clades defined by DNA fingerprinting by Ca3 hybridization, except for clade E (34). Based on the results, MLST possesses four well defined clades, designated 1 to 4. It is important to mention that an arbitrary *p* distance cutoff was employed to delineation of *C. albicans* clades. A minority of isolates can shift to different clades as data set grows. However, the largest clade groups are stable (34).

Tavanti *et al* (56) developed an interesting study analyzing a collection of 416 *C. albicans* isolates that clustered into 11 groups by MLST, named from 1 to 11. Fifty percent of the isolates belonging to group 5 were resistant to Fluconazole and Itraconazole and isolates that demonstrated resistance to flucytosine were concentrated into groups 1, 5 and 6. However, the number of isolates was too low to allow definitive conclusions.

The correspondence between the clade systems developed by MLST and Ca3 hybridization is remarkable, because epidemiological studies performed with both methodologies can be compared. Regarding the anatomical sites, the proportion of isolates from blood and other sterile sites that belonged to clade I was lower than in the other major clades. Considering clade I isolates, Four out of five isolates belonged to cluster 1 when eBURST algorithm analysis was performed. Similarly, clade II isolates were related to cluster 2, clade SA isolates corresponded to clade 3, and clade E isolates to clade 4. Clade III isolates, as determined by Ca3 hybridization, corresponded to clusters 9 or 10 when analyzed by eBurst algorithm. In a comparable study, Odds *et al* (32) evaluated 1391 isolates and demonstrated that 97% of them could be grouped in one of the 17 groups or clades based on their MLST profile. Groups 1 to 4 comprised the majority of the isolates and these groups corresponded to clades I, II, III and SA respectively, as determined by Ca3 hybridization. Similarly, Clade 11 corresponded to clade E. This study also established that the proportions of A, B, and C genotypes, defined by the presence or absence of an intron in the ribosomal DNA region, differed significantly among clades. In this work, 93% of strains in clades 1 and 2 were type A. Furthermore, North American isolates were predominantly assigned as clades 1 and 3 and 40% of African isolates were grouped into clade 4.

The studies mentioned above demonstrated that results obtained by MLST can be compared with those obtained by Ca3 hybridization, although the correlation could not be completely demonstrated for all clades. This is an interesting point, due to the possibility of using the results obtained in epidemiological studies. Considering the practicability of the MLST experimental performance and analysis, it is considered as the method of choice for *Candida* genotyping, which has been largely contributing to the understanding of the evolutionary origins of *Candida albicans* different strains.

Microsatellites analysis

Microsatellites are short tandem repeated sequences interspersed randomly through the nuclear and organelles genome, and basically the repeating unit consists of fragments shorter than 10 bases pair (bp). Between the individuals the repeating units number found in the microsatellite varies, which contributes with allele polymorphisms. Besides, the microsatellites are quite unstable and high polymorphic due to mispairing slippage during replication, which lead to their expansion or contraction, and also point mutations inside or outside the repeated region in the genomic structures (53). Interesting they present mendelian codominant inheritance and PCR typing simplicity which make them useful to identify hereditary relationships and for genotyping microorganisms. Microsatellite DNA typing can provide important correlations between genotypes and location of infection, degree of virulence, or drug susceptibility. Such correlations are crucial for proper, large-scale epidemiological analysis (53).

Using microsatellites as molecular markers for microorganisms' genotyping, several polymorphic microsatellite loci were identified in the genome of *C. albicans* (25). Sampaio *et al*. (46) described a microsatellite locus (CAI) in a non-coding region in the genoma of *C. albicans* and evaluated its applicability to accurately differentiate strains. The discriminatory power of this locus is the highest for any loci tested. Liu *et al*. (25) developed a study employing polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) of CAI microsatellite for genotyping of vulvovaginitis *C. albicans* isolates. The molecular results showed a correlation between the groups characterized and their antifungal susceptibility profile. The isolates belonging to the dominant genetic related groups (named as groups A, B, C, and D) were susceptible to amphotericin B, flucytosine, ketoconazole and fluconazole. *C. albicans* genotype A was less resistant to azole antifungals, and *C. albicans* genotype B was more resistant to itraconazole. Also, Fan *et al*. (19) reported that the frequency of *C. albicans* with the dominant genotypes (A to D) from patients with severe vulvovaginal candidiasis was significantly higher than that from patients with mild-to-moderate vulvovaginal candidiasis. This suggested that the CAI genotype distribution of *C. albicans* strains is correlated with the severity of vulvovaginal candidiasis. Typing of independent *C. albicans* strains from sputum and other extragenital clinical samples indicated that few of the strains had the same or similar CAI patterns. These researchers suggested that the distribution of the dominant genotypes in the *C. albicans* strains from the vaginas of asymptomatic women, from extragenital sites, especially the gastrointestinal tracts and oral cavities of women with vulvovaginal candidiasis and from the penises and oral cavities of men will be helpful in elucidating the source of the infection.

***Candida* clades and phenotypic variations**

The identification of major *Candida* clades has lead to the investigation of characteristics that could be related to each group. Since clades persist in the same geographic area and recombination is not a frequent event, the evolutionary process inside each clade may occur independently, and this fact may have contributed with the development of specific phenotypes. According to Soll and Pujol (55), every aspect of *C. albicans* biology and physiology should be reassessed in this basis.

In Pujol *et al* (41) the authors demonstrated that natural resistance to flucytosine was restricted to group I isolates; 96% of the isolates studied had increased minimum inhibitory concentrations for flucytosine belonged to group I. Dodgson *et al* (17) showed that both decreased susceptibility and increased

resistance correlate in the majority of cases with a point mutation from cytosine to thymine at position 301 of the gene *FUR1*, which encodes phosphoribosyltransferase. This change results in an amino acid substitution from arginine to cysteine at position 101 in the Fur1 protein. The authors also showed that the mutation was restricted to clade I strains.

Considering the phenotypic analysis between the strains, Blignaut *et al* (3), using Ca3 hybridization to fingerprint 38 amphotericin B-resistant in *C. albicans* isolates, reported that the yeasts clustered in all clades, except clade III. Similarly, Liu *et al*. (25) associated the dominant groups found by microsatellite analysis with susceptibility profile to antifungal agents. However, the inexistence of studies that correlates the major groups formed by CAI microsatellite genotyping with the clades grouped by Ca3 hybridization or with MLST limits the analysis of *Candida* clades and their correlation with the phenotypic characteristics.

Several virulence factors contribute to the pathogenicity of *Candida* yeasts, including the ability to adhere to epithelial and endothelial cells, to hyphal germination, to extracellular proteinases and phospholipases, and to phenotypic switching (20, 26). Genotyping methods have been employed to investigate the correlation of strains and their virulence, but in many times, the methods employed lacked in accuracy and provided few information (54). Schmid *et al* (50) performed the clustering of 47 *Candida* isolates into 22 groups and attempted to correlate these groups with physiological traits in order to explain characteristics that determine colonization or infection. The authors found a great predominance of one group (group 13), which demonstrated the ability of the members of this group in colonizing human individuals. It was also observed that the members of this group were more often resistant to five fungicidal substances tested and that those isolates adhere more strongly to saliva-coated surfaces. The authors suggested that these characteristics may promote the pathogenicity of *C. albicans.*

The *ALS* (agglutinin-like sequence) gene family of *C. albicans* encodes eight large cell-surface glycoproteins, some of which act in adhesion process to host cells (35). The protein Als3p produced on *C. albicans* germ tubes and hyphae demonstrated to have adhesive function. Disruption of *ALS3* gene results in cells with reduced adherence to vascular endothelial and buccal epithelial cells, as well as a marked reduction in destruction of epithelial cells in the reconstituted human epithelium in *in vitro* model of candidiasis (20). Considering this, Zhao *et al* (60) developed a program with the aim of verifying the occurrence of clade-specific differences regarding *ALS3* genes. Clades differed with respect to prevalent *ALS3* alleles and its distribution, but were similar for the mean number of tandem repeat copies per *ALS3* allele. One allele (*ALS3*(*12*)) has 12 tandem repeat copies while the other (*ALS3*(*9*)) has 9 copies (35). Other studies related to the ALS gene family and their ability to influence cellular adhesion were also performed by the same research group (61). Using genomic DNA from *C. albicans* isolates from different geographical region, it was PCR-amplified and they demonstrated that prevalent alleles and allelic distributions varied among the clades for *ALS5* and *ALS6.* The authors also observed that ALS6 exhibited less variability than *ALS5* and the deletion of both *ALS5* alleles was associated particularly with clades III and SA.

Virulence factors of *C. albicans* and resistance to new antifungal agents are aspects that should be reevaluated by correlating the phenotypic characteristics with DNA fingerprinting. Researches have been collaborating to a more wild comprehension about these topics when they use genotyping methods that can be compared with other results in order to form a global profile of virulence related to strain specificity.

CONCLUSION

The present review presented a brief description of methods employed in *C. albicans* genotyping, with special emphasis to Ca3 hybridization, MLST and Microsatellites. The arrangement of *C. albicans* yeasts in clades or groups provided important information about this organism regarding the geographic maintenance of strains and in the mode of

reproduction, which is probably clonal with certain degree of DNA rearrangement that contributes to yeast microevolution. Virulence factors and antifungal resistance can be associated to established clades, but a broad field of investigation must be explored; several physiological traits should be reevaluated on this basis.

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