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Molecular Methods Developed for the Identification and Characterization of *Candida* Species

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Abstract

Candida species are commensally microorganisms in healthy individuals, but is also the most opportunistic fungal pathogen of human. From benign skin-mucosal forms to invasive ones, *Candida* infection, can compromise various organs systematic and cause diseases in immunocompromised or critically ill patients. Hence the accurate identification and characterization of the disease-causing strains is crucial for diagnosis, clinical treatment and epidemiological studies of candidacies. Molecular methods the identification and characterization support gives in epidemiological research and in development of novel antifungal with specific molecular targets, especially in the case of resistant *Candida* species. This review aims to describe the main methods used in the identification and characterization the *Candida* species and discusses future perspectives.

Keywords: Candidiasis; ITS; Genomes; Molecular typing

Introduction

Candida species commensally inhabit the human body. The relationship between an autochthonous *Candida* species and its human host can be affected by pathological, physiological, mechanical and iatrogenic factors. In this sense, *Candida* species can cause several types of infections with a wide spectrum of clinical presentations, from superficial benign forms to invasive ones that compromise several organs, leading to host death [1]. *Candida* species exhibit a large clinical relevance, therefore is necessary to correctly identify and characterize the isolates at the molecular level for understanding the spread of species and the mechanisms of their resistance to antifungal agents [2].

The resistance of *Candida* species to antifungal agents can often be attributed to a mutation gene or other alteration (such as increased expression) of the drug target [3]. In this sense, were developed several effective molecular methods for the identification and characterization of *Candida* species. These methods support gives in epidemiological research and in development of novel antifungal with specific molecular targets, especially in the case of resistant *Candida* species. This review summarizes the insights the main methods used in the identification and characterization the *Candida* species and discusses future perspectives: omics technologies for characterization of *Candida* species.

General features: *Candida* species

Candida species are taxonomically classified in the kingdom Fungi, phylum Ascomycota, class Saccharomycetes, family Saccharomycetaceae, and genus *Candida*. These yeasts are unicellular microorganisms that are pleomorphic and ovoid or spherical in shape with an incomplete sexual cycle. *Candida* species commensally inhabit the human body and can be found in the respiratory tract, gastrointestinal tract, vaginal mucosa, oral cavity, and skin of healthy individuals [4]; they can also be isolated from plants, water, soil, and other environments. Moreover, they can degrade proteins and carbohydrates as a source of carbon and nitrogen, which are essential for their development [5].

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There are close to 200 different *Candida* species, five of which *Candida albicans, Candida tropicalis, Candida glabrata, Candida krusei,* and *Candida parapsilosis* [subdivided into *C. parapsilosis, Candida orthopsilosis*, and *Candida metapsilosis*] (Figure 1) are involved in more than 90% of invasive infections [6,7]. Other emergent *Candida* species, such as *Candida guilliermondii, Candida dubliniensis, Candida lusitaniae, Candida kefyr, Candida rugosa, Candida famata, Candida utilis, Candida lipolytica, Candida norvegensis*, and *Candida inconspícua*, also have clinical relevance, and have been identified as causative agents of superficial and systemic itches [7,8].

C. albicans is considered the most frequently isolated species from patients with superficial and invasive infections of different

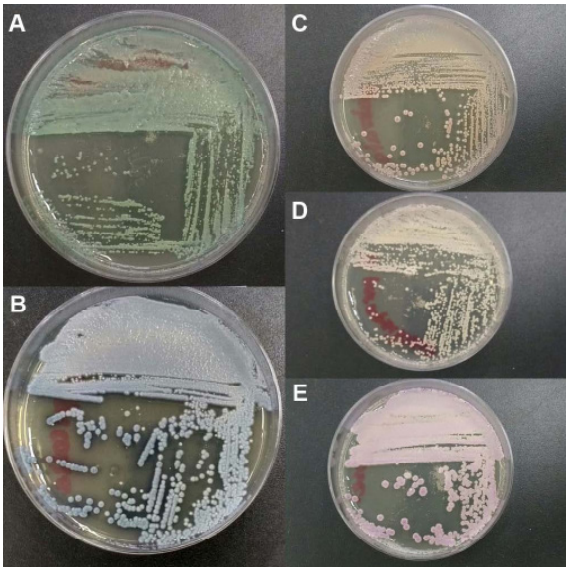

Figure 1: *Candida* species clinical, isolated on selective and differential CHROMagar™ *Candida* DIFICO®. A) *Candidaalbicans*; B) *Candidatropicalis;* C) *Candidaglabrata;* D) *Candidaparapsilosis*; E) *Candidakrusei.*.

anatomical sites in case studies from around the world [9]. Its main pathogenic mechanisms and virulence factors appear to be its ability to adhere to different mucous membranes and epithelia, its ability to produce filamentous structures that assist in tissue invasion, and its production of enzymes such as proteinases and phospholipases. Such species are naturally sensitive to antifungal agents when used systemically, but cases of acquired resistance to azolics, in particular to fluconazole, have been described in patients that receive long-term antifungal therapy [10].

In Latin American countries, especially Brazil, *C. tropicalis* is detected in 20-24% of blood-borne infections [10,11], mainly in elderly patients and those with conditions such as neutropenia and Diabetes mellitus [12]. Clinical isolates of this species are typically sensitive to amphotericin B and triazoles [13]; however, cases of resistance to these drugs, especially to fluconazole, have been recently reported and described [14].

The species *C. glabrata* and*, C. krusei* also have pathogenic potential [10,11]. *C. glabrata* ranks second among isolates from blood-borne infections in the United States. This species has also experienced an increase in the percentage of fluconazole-resistant isolates as well the incidence of isolates with reduced sensitivity to amphotericin B and cross-resistance to other drugs from the azole class [10]. *C. krusei* has been shown to be an occasional hospital pathogen, particularly in patients with hematological malignancies and/or that undergoing bone marrow transplantation. *C. krusei* is naturally resistant to fluconazole [15].

C. parapsilosis has been isolated from health professional studies and parenteral nutrition solutions [16]. This yeast has been recognized as a major cause of candidemia related to infections that begin from the skin [17]. In general, clinical isolates of this species are sensitive to most antifungal agents, especially amphotericin B and azoles. However, in clinical studies, isolates with reduced sensitivity to fluconazole have been reported [18]. Together, *Candida* species exhibit a large clinical relevance due to their incidence of colonization and infection of the human body. It is therefore necessary to correctly identify and diagnose the yeast species responsible for an infection and subsequently prescribe the proper antifungal agent for treatment.

Phenotypic methods used in the identification and characterization of *Candida* species are based on analysis of morphological and biochemical profile of these organisms. The most widely used phenotypic methods for identification and characterization the *Candida* species are observation of microscopic structures, evaluation tests of enzyme activity and assimilative capacity and substrate fermentation [19].

Several commercial products and systems have been developed the aim of solve some difficulties experienced by the clinical microbiology laboratories in the diagnosis of infections caused by *Candida* species [20], for example Agar containing chromo gens, kits and panels semi or full automated to the presumed or definitive identification of the most prevalent species [21,22]. The use of only phenotypic testing is not highly effective for identification of *Candida* species because some species has few morphological and biochemical variations, therefore phenotypic and molecular methods can be used in together to increase reliability in the identification of these species.

Identification and Molecular Characterization

In recent decades, molecular biological techniques have been used to better understand the pathogenicity of *Candida* species and to expand the search for new molecular drug targets. *Candida* species are diploid, heterozygous, and contain a plastid genome, features which recent genomic approaches have been able to better characterize. In 1996, the *Candida Genome Sequencing Project* was initiated with a goal of sequencing the genome of *C. albicans* SC5314 [23,24]. This goal necessitated the use of bioinformatics tools that were able to predict and annotate genes in the sequence. The result was the description of 6,354 genes, although the DNA sequences of some specific chromosomes had still not been determined.

Comparison of the *C. albicans genome* with the genomes of other fungal species allowed for the identification of several specific genes that could be potential targets for antifungal therapies. It was observed that compared with other fungi, the coding sequence of the *C. albicans genome* was rich in short tandem repeats (STRs). It was also possible to identify and conduct detailed analyses of multi genic families found in *C. albicans*, many of which were related to its pathogenicity [23].

In 2007, Van Het Hoog, et al. [25] provided the complete sequence of the *C. albicans* SC5314 genome (15.845 Mb organized into 8 chromosomes). In addition to its utility for genetic mapping, it provided updates for certain genome characteristics, including the discovery of an additional transcription factor gene family, information concerning the chromosomal locations of gene families, and a review of the open reading frame (ORF) list previously annotated by Braun, et al. [23].

To determine the genetic characteristics underlying the biological and pathogenic diversity of the different *Candida* species, Butler, et al. [26] proposed sequencing of *C. albicans* (WO-1), *C. tropicalis*, *C. parapsilosis*, *C. guilliermondii*, *C. lusitaniae*, and *Lodderomyceselongisporus* (a close relative of *C. parapsilosis*) along with subsequent comparisons to the already sequenced genomes of *C. albicans*SC5314 and *Debaryomyceshansenii*, a marine yeast rarely associated with disease [23-25,27].

The genomes of the species sequenced by Butler, et al. [26] ranged in both size (between 10.6 to 15.5 Mb) and composition of protein-coding genes (5,733–6,318 genes) across the different species. The authors also identified 64 gene families that appeared to be related to *Candida* pathogenicity. Six of these families had been previously shown to be associated with pathogenicity, including the ERG3 gene, which is involved in the ergosterol biosynthesis pathway. The dissemination of *C. albicans* and Non-*albicans Candida* (NAC) genomes in the public domain accelerated research on the biological and molecular mechanisms associated with the pathogenicity of these species.

Due to the fact that some species of *Candida* present few easily identifiable morphological and biochemical variations, molecular biological techniques have been used to overcome the limitations of phenotypic identification methods. Fungal molecular systematic is based primarily on the analysis of mitochondrial genes (mtDNA) and ribosomal DNA (rDNA) [28].

The mitochondrial cytochrome C oxidase subunit 1 (*COI*) was proposed for molecular identification at the species level [29]. This gene was adopted by the Consortium for the Barcode of Life for the classification of all organismal groups, including fungi [30]. *COI* works reasonably well as a barcode in some fungi genera, such as *Penicillium* [31]; however, results in other groups that have been experimentally examined are inconsistent, limiting this gene's use [32].

Ribosomal genes are conserved among all known organisms. As a consequence, this gene allows for the joint reconstruction of both prokaryotic and eukaryotic phylogenies. On the other hand, the nucleotide substitution rate is low in the 18S rRNA gene [33], often preventing discrimination between closely related species. Thus, species that exhibit differences at the ribosomal gene level have likely already been diverging for at least a few million years.

For discrimination of closely related filamentous fungal species and yeasts, the Internal Transcribed Spacer regions (ITS 1 and ITS 2), located between the 18S, 5.8S, and 28S rRNA genes, and the D1/D2 region, located in the larger rDNA subunit, have shown to be successful in species-level identification (Figure 2). In both ITS1 and ITS2, there are 100-200 tandem repeats, which contain both highly conserved domains and variable domains, respectively [34]. These regions have been adopted for use in bar-coding for most fungi genera and are considered standard markers by the Consortium for the Barcode of Life [35].

Kurztzman, et al. [36] described the presence of extensive genetic differentiation in the D1/D2 region, allowing for the differentiation of ascomycetes. Since then, this region, together

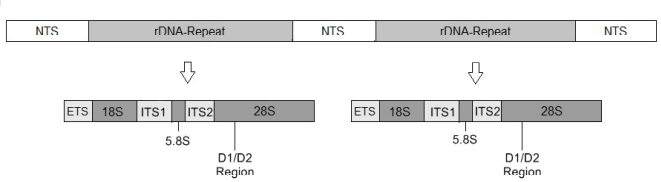

Figure 2: Structure of fungal DNA. NTS: Non- transcribed spacer, ETS: External transcribed spacer, ITS: Internal transcribed spacer, genes 18S, 5.8S and 28S ribosomal the DNA.

with the ITS regions, have been widely used for both identification and establishment of evolutionary relationships (phylogenies) of several fungi species, including *Candida* species. Phylogenies inferred from molecular data have been used to clarify the main evolutionary divisions between taxa, as well as helping in the identification process of higher taxonomic groups, including the Fungi kingdom [28].

Evolutionary histories as elucidated by phylogenetic analysis are normally illustrated as branching, treelike diagrams that represent estimates of inherited relationships between molecules (phylogenetic tree), organisms, or both [37]. There are three classes of phylogenetic methods: distance-based, character-based, and Bayesian inference-based. In models that use distances for phylogenetic reconstruction, two steps are necessary: distance calculation and construction of the topology.

For distance-based calculation, calculated matrices are used for pair wise comparisons between the aligned sequences based on a replacement model, i.e., an evolutionary model of these sequences. Among the most widely used models for phylogenetic analysis in *Candida* are the P Distance, Juckes-Cantor, Kimura 2-parameter, Tajima and Nei, Tamura 3-Parameter, Tamura and Nei, Gamma-Poisson, and PAM Distance models. The most common algorithms used to order the calculated distances between the macromolecular sequences contained in the matrix into a topology are UPGMA (Unweighted Pair Group Method with Arithmetic Mean), Neighbor-Joining, and Minimum Evolution [38].

The character-based models are directly inspired by the cladistics methods of Maximum Parsimony and Maximum Likelihood [39]. For models based on Bayesian inference, the parameters are considered random variables in which the uncertainty about values is measured by the distribution of posterior probabilities [40].

Many techniques are used for molecular characterization of *Candida* species, such as Restriction Fragment Length Polymorphism (RFLP) [41], DNA fingerprinting [42], electrophoretic karyotyping [43], Random Amplified Polymorphic DNA (RAPD) [44], Multilocus Enzyme Electrophoresis (MLEE) [45], and sequencing of microsatellites [46]. However, in order to estimate genetic distances and infer phylogenetic relationships between species that can be easily evaluated in terms of probability models, the most suitable techniques involve gene sequencing and produce topologies built from nucleotide or amino acid sequences.

One of the techniques used for molecular characterization are the microarrays. Microarray-based systems offer an attractive outlook for the of strain typing. They can offer high level of specificity, sensitivity and through put capacity. For typing molecular, microarrays can be used to identification and get the different sequence variants of specific genes or regions and ITSs, in particular. Ongoing sequencing projects in pathogenic yeasts also enable quite straightforward designing of whole-genome DNA microarrays [47].

Multilocus Sequence Typing (MLST), a sequencing-based method, was initially developed for both clone identification and bacterial pathogen typing [48]. This method analyzes nucleotide polymorphisms in essential genes fragments, the "housekeeping genes," and can produce sequences of up to 500 bp [48-50], generating a molecular characterization with high discriminatory power and reproducibility.

The MLST technique has been used previously for several *Candida* species, among them, *C. albicans* [49,51]. Based on collaborative work, a set of seven essential *C. albicans* genes were proposed for the analysis [52]. This set includes *AAT1a*, *ACC1*, *ADP1*, *SYA1*, *VPS13*, *ZWF1b*, and *MPIB,* which has since been renamed *PMI1* [53]. MLST proved to be a useful method for epidemiological differentiation of clinical isolates of *C. albicans* [49,51].

Tavanti, et al. [54] found MLST on a panel of 416 isolates of *C. albicans* from different sources recognized a population structure comprising four major clades and eight minor clades. Odds, et al. [55] evaluated larger panel of *C. albicans* isolates (1391) for MLST analysis, the number of clades recognized increased to clades 17. ABC types (based on the presence or absence of an intron in rDNA) and geographical origins showed statistically significant variations among clades, but anatomical source and antifungal-susceptibility data were not significantly associated. Computational haplotype analysis of the gene fragments sequenced for MLST showed a high frequency of recombination events, which suggests that *C. albicans* isolates had mixed evolutionary histories resembling those of a sexually reproducing species [55]. Also mitochondrial genes have been demonstrated by Wang, et al. [56], to be promising targets for genotyping and population genetics of *C. albicans.*

Ge, et al. [57] evaluated *C. albicans* isolates from the vagina and oral cavity of Chinese candidose vulvovaginal patients and asymptomatic carriers. The genotypes of these strains were identified. Antifungal susceptibility testing revealed that the two dominant genotypes, CAI 30–45 and CAI 32–46 associated with vulvovaginitis showed significantly different azole-susceptibility. Different mutation patterns in the azole target gene ERG11 were correspondingly observed among *C. albicans* isolates representing different genotypes and sources. Isolates with the same or similar CAI genotypes usually possessed identical or phylogenetically closely related ERG11 sequences. Loss of heterozygosity in ERG11 was observed in all the CAI 32–46 isolates but not in the CAI 30–45 isolates and most of the oral isolates sequenced. Compared with the ERG11 sequence of strain SC5314, two homozygous nonsynonymous substitutions, leading to two amino-acid changes (A114S and Y257H) in the Erg11p were found in CAI 32–46 isolates. The association between azole-susceptibility and *C. albicans* genotype may be of potential therapeutic important.

Dodgson, et al. [58] developed MLST for *C. glabrata* through amplification and sequencing of fragments from the coding regions of six genes (*FKS*, *LEU2*, *NMT1*, *TRP1*, *UGP1,* and *URA3*). An MLST analysis of 230 *C. glabrata* isolates from five populations that differed both geographically and temporally confirmed that using the six loci, it was possible to assess genetic diversity and differentiation among isolates of this species [59].

Tavanti, et al. [60] described a high degree of reproducibility and discriminatory power above 99% when using MLST to differentiate between isolates of *C. tropicalis* through sequencing of polymorphic fragments of six genes (*MDR1*, *ICL1*, *SAPT2*, *SAPT4*, *XYR1*, and *ZWF1a*). The method could differentiate between 87 of the 106 DST isolates tested. Jacobsen, et al. [61] later conducted a MLST study of the *C. tropicalis* molecular phylogeny with 242 isolates; the haplotype analysis revealed several recombination events.

While conducting a molecular characterization study of 61 isolates of *C. tropicalis*, Magri, et al. [62] found that only 3 isolates were resistant to fluconazole, but it was not possible to correlate the Diploid Sequence Types (DSTs) with resistance. These same authors reported that MLST is an important tool for the study of genetic diversity, particularly with regard to polymorphisms. Although many molecular studies have been conducted to analyze the different *Candida* species, more research is needed to investigate the genetic and phylogenetic diversities of *Candida* species and their relation to epidemiology. The development of next-generation sequencing (NGS) over recent years has allowed a technological breakthrough in the molecular characterization of *Candida* species. This technology allows accurate and thorough genotyping of genes involved in antifungal resistance in strains clinical. Garnoud, et al. [63] used NGS to investigate echinocandin and azole resistance in clinical *Candida* isolates. Six genes involved in antifungal resistance (ERG3, ERG11, TAC1, CgPDR1, FKS1 and FKS2) were analyzed in 40 *Candida* isolates (18 *C.albicans*, 15 *C.glabrata* and 7 *C.parapsilosis*). A total of 391 SNPs were detected, among which 6 coding SNPs were reported for the first time. Novel genetic alterations were detected in both azole and echinocandin resistance genes. A *C. glabrata* strain, which was resistant to echinocandins but highly susceptible to azoles, harboured an FKS2 S663P mutation plus a novel presumed loss-of-function CgPDR1 mutation. Another *C. glabrata* isolate, carried a new FKS2 S663A mutation and a new putative gain-of-function CgPDR1 mutation (T370I). This study shows that NGS can be used for extensive assessment of genetic mutations involved in antifungal resistance.

Genome sequencing of *Candida* species provides the opportunity to elucidate some of the mechanisms involved in intrinsic or acquired resistance by yeasts of the *Candida* genus. The omics technologies (genome, transcriptome, proteome,

metaboiome, microbiome and mycobiome) will become very valuable for detecting mechanisms of resistance in clinical strains subjected to multidrug pressure. The high-throughput sequencing methods still has little practicability and feasibility in daily clinical practice and thus still remains challenging, but as future perspectives, current rapid progression of automation of these technologies makes their upcoming routine application likely.

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