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REVIEW ARTICLE

Molecular fingerprinting methods for the discrimination between *C. albicans* and *C. dubliniensis*

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Opportunistic fungal pathogens are becoming increasingly important causes of both community-acquired and nosocomial infections. The most important fungal pathogens are yeast species belonging to the genus *Candida*. These species show differences in levels of resistance to antifungal agents and mortality. Consequently, it is important to correctly identify the causative organism to the species level. Identification of *Candida dubliniensis* in particular remains problematic because of the high degree of phenotypic similarity between this species and *Candida albicans*. However, as the differences between both are most pronounced at the genetic level, several studies have been conducted in order to provide a specific and rapid identification fingerprinting molecular test. In most candidal infectious, no single DNA fingerprinting technique has evolved as a dominant method, and each method has its advantages, disadvantages and limitations. Moreover, the current challenge of these techniques is to compile standardized patterns in a database for interlaboratory use and future reference. This review provides an overview of most common molecular fingerprinting techniques currently available for discrimination of *C. albicans* and *C. dubliniensis*.
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Introduction

Opportunistic fungal infections have gained substantial importance during recent years and yeasts, primarily *Candida* species, have been the most common fungi isolates from human infections (Hazen, 1995). The number of life-threatening forms of candidosis has increased considerably, with attributable mortality as high as 38% (Wey *et al*, 1988) and crude mortality rates exceeding 50% (Wenzel, 1995). *Candida* species causes superficial mucosal infections or disseminated forms of diseases such as community-acquired and nosocomial infections (Sullivan *et al*, 1997). The risk factors that predispose to these infections include immunocompromised patients exposure to treatments such as multiple antibiotics, chemotherapy, central catheterization, immunosuppressive therapy and anti-retroviral therapy (Hazen, 1995; Wenzel, 1995; Willis *et al*, 2000).

Although *Candida albicans* remains the most frequent cause of oral candidosis (Coleman *et al*, 1999), the incidence of disease caused by other species of *Candida* has been increasing steadily (Coleman *et al*, 1997a, 1999). One possible explanation is the increased use of azoles, which has positively selected for some less sensitive *Candida* species such as *Candida dubliniensis* (Moran *et al*, 1997). Since *C. dubliniensis* was first isolated from oropharyngeal lesions in patients with AIDS (Sullivan *et al*, 1995), it has been reported from patients worldwide (Boerlin *et al*, 1995; Pujol *et al*, 1997; Sullivan *et al*, 1997; Salkin *et al*, 1998; Alves *et al*, 2001). Although most of the *C. dubliniensis* isolates have been identified from the oral cavities of HIV-infected patients (Sullivan *et al*, 1995, 1997), it has also been isolated from other anatomical sites, including lungs, vagina, blood, and feces (Moran *et al*, 1997; Odds *et al*, 1998; Sullivan and Coleman, 1998; Meis *et al*, 1999). Moreover, *C. dubliniensis* can be a constituent of normal human oral flora, with the potential to cause oral candidosis (Coleman *et al*, 1997a; Sullivan *et al*, 1997). These isolates have also been recovered from blood, gastrointestinal tracts and vaginas of HIV-negative patients, including healthy, diabetic and bone marrow transplant individuals (Moran *et al*, 1997; Pinjon *et al*, 1998; Meis *et al*, 1999). McCullough *et al* (1995) have

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shown that *C. dubliniensis* isolates may be more virulent, as they have significantly higher levels of proteinase activity and greater levels of adherence to buccal epithelial cells than do typical *C. albicans* strains. It has been demonstrated that although most isolates of *C. dubliniensis* are sensitive to the commonly used antifungal agent fluconazole (Moran *et al*, 1997; Kirkpatrick *et al*, 1998), a significant proportion of isolates can rapidly develop stable resistance after exposure to the drug *in vitro* (Moran *et al*, 1997). Therefore, immunocompromised patients who have received multiple treatments for fungal infections may be at increased risk for harboring *C. dubliniensis* as the predominant species in their oral cavities (Jabra-Rizk *et al*, 1999).

Retrospective studies have recently shown that some previously diagnosed *C. albicans* infections were actually *C. dubliniensis* infections (Odds *et al*, 1998; Sullivan and Coleman, 1998). These species share many phenotypic characteristics, including resistance to cycloheximide and ability to produce germ-tubes, chlamydospores and true hyphae (Sullivan *et al*, 1995; Sullivan and Coleman, 1998). Although *C. dubliniensis* and *C. albicans* may differ in their carbohydrate assimilation profiles, growth patterns at elevated temperatures, and intracellular β-glucosidase activities (Coleman *et al*, 1997a; Pinjon *et al*, 1998; Salkin *et al*, 1998), to date none of these methods appear to definitively identify the two species (Sullivan and Coleman, 1998; Campanha *et al*, 2005). As epidemiology, virulence and antifungal susceptibility often vary among strains, a rapid and accurate identification of the disease-causing isolates of *C. albicans* and *C. dubliniensis* is crucial for clinical treatment and epidemiological studies. Nevertheless, because the differences between *C. dubliniensis* and *C. albicans* are most pronounced at the genetic level (Coleman *et al*, 1997a; Sullivan and Coleman, 1998) several studies have been conducted in order to provide a specific and rapid identification method for the identification and typing these organisms. Molecular fingerprinting methods differ in their taxonomic range, discriminatory power, reproducibility, and degree of interpretation (Vaneechoutte, 1996; Taylor *et al*, 1999; Soll, 2000). In most candidal infectious, no single molecular fingerprinting technique has evolved as a dominant method, and in fact, each method has its own set of assets and limitations (Sullivan *et al*, 1996; Vaneechoutte, 1996; Williams and Lewis, 2000). Thus, the main objective of this review was to describe the principles, advantages, disadvantages and limitations of most common molecular fingerprinting techniques currently available for discrimination of *C. albicans* and *C. dubliniensis*.

The most common methods to DNA fingerprinting C. albicans and C. dubliniensis

Electrophoretic karyotyping

A suitably high level of candidal discrimination has been reported using electrophoretic karyotyping (EK) analysis suggesting that the technique can be of value in epidemiological typing of *Candida* strains (Williams and Lewis, 2000). In general technique, cells are mixed with enzymes to remove the cell wall and afterward embedded in an agarose plug. Protease and detergent are added, and the cells are incubated to remove membranes and digest protein. The agarose plug is placed in a well at the top of an agarose slab gel, and electrophoresis is conducted following the procedures outlined by particular separating system (Zolan, 1995; Soll, 2000). Chromosomal DNA bands are resolved by electrophoresis separation according to size in agarose gels and visualized by ultraviolet (UV) transillumination after ethidium bromide staining. Pattern differentiation may be carried out by visual comparison (Sullivan *et al*, 1996). Specific chromosomes can be identified using Southern blot hybridization with chromosome-specific DNA probes (Soll, 2000). The development of pulsed-field gel electrophoresis (PFGE), orthogonal-field-alternation gel electrophoresis (OFAGE), and variations upon these basic electrophoretic karyotyping systems such as contour-clamped homogeneous electric field gel electrophoresis (CHEF) and transverse alternating-field electrophoresis (TAFE) permitted the electrophoretic separation of chromosome-sized of the yeasts DNA molecules and also the demonstration of frequent chromosome-length polymorphisms (Merz, 1990; Sullivan *et al*, 1996; Höfling *et al*, 1997; Soll, 2000; Williams and Lewis, 2000).

The PFGE bases on the concept that the limit of the size of DNA separable by conventional electrophoresis in agarose (over 50 kb) may be enlarged by introducing pulses or changes in the direction of the electric field subjecting the DNA to an electrophoretic field that alternates in its direction (Sullivan *et al*, 1996; Höfling *et al*, 1997). Larger DNA molecules require greater time to re-orient than smaller molecules thus enabling resolution (Williams and Lewis, 2000). Sullivan *et al* (1995) used phenotypic tests followed by molecular methods to describe *C. dubliniensis* as a new candidal species. Ten atypical isolates yielded karyotype profiles with one or more chromosome-sized bands of <1 Mb, a feature also displayed by the reference type I *C. stellatoidea*, but not by *C. albicans*. These data combined with phenotypic characteristics and other molecular methods suggested that these atypical organisms constituted the new species *C. dubliniensis*. Likewise, other studies used EK by PFGE to confirm the discrimination between *C. albicans* and *C. dubliniensis* suggested by phenotypic tests (Jabra-Rizk *et al*, 1999). In an epidemiological study of *Candida* colonization in HIV-patients (Millon *et al*, 2002), molecular typing methods were compared with internal transcribed spacer (ITS) analysis. EK by PFGE and RAPD showed that one patient was co-infected by two distinct genotypes and ITS analysis identified one of two genotypes as *C. dubliniensis*. Interpretation of EK pattern by visual comparison was easy because of the good resolution and high intensity of the bands. However, strains presenting minor EK variations were difficult to classify because these variations could be interpreted either as strain replacements or as chromosomal rearrangements in a single strain. Diaz-Guerra *et al* (1999) used EK analysis to identify the recurrent isolation of *C. dubliniensis* from two HIV-infected patients with oral candidosis and undergoing

azole therapy. According to the authors, EK analysis was able to distinguish *C. dubliniensis* isolates from the *C. albicans* strains.

The EK has been demonstrated as a molecular tool of good resolving power and high reproducibility for typing of *Candida* strains, including *C. albicans* and *C. dubliniensis* (Diaz-Guerra *et al*, 1999; Millon *et al*, 2002). However, some practical drawbacks may limit the value of this method as a diagnostic tool in routine laboratories. These limitations include the need for specialized equipment, the small number of isolates that can be included on a single gel, prolonged turnaround time (about 5 days), and the need to include both control and unknown isolates on the same gel to detect method-related differences in mobilities between runs (Sullivan *et al*, 1996; Soll, 2000; Williams and Lewis, 2000). Moreover, further studies are warranted to evaluate the use of other variations upon basic electrophoretic karyotyping such as OFAGE and TAFE for discrimination between *C. albicans* and *C. dubliniensis*.

Restriction fragment length polymorphism

The basic principle of the restriction fragment length polymorphism (RFLP) technique consists of the analysis of fragments obtained by restriction endonuclease digestion of genomic DNA from a species and separation of the fragments by agarose gel electrophoresis (Scherer and Stevens, 1988). Two methods are commonly used to detect differences or similarities in the fragments (Merz, 1990). In the first method, the profiles can be visualized directly by UV transillumination after staining with ethidium bromide labeling specific fragments with DNA probes (Merz, 1990; Williams and Lewis, 2000). Afterward, the banding patterns are analyzed. Multiple bands can be detected, and bright bands representing DNA sequences repeated in the genome can easily be seen; differences in mobilities can also be determined (Merz, 1990). The second method is detection of fragment length polymorphisms of a specific DNA sequence by DNA hybridization with species-specific DNA probes (Scherer and Stevens, 1988; Merz, 1990). Generally, the technique of hybridization used for identification of *Candida* species is performed as described by Southern (Southern, 1975). In this technique, the digested DNA present in the RFLP gels is transferred by capillary blotting to nylon membranes and hybridized with species-specific probes, radioactively labeled by random priming and exposed to autoradiography film (Merz, 1990). Then, the banding patterns visualized on the autoradiographs are analyzed. Single bands of similar or different length or multiple bands may be detected and compared among isolates. In RFLP techniques, the banding patterns can be analyzed by both visual interpretation and computerized programs (Scherer and Stevens, 1988; Merz, 1990; Williams and Lewis, 2000).

The results of a study by Sullivan *et al* (1995) showed that the restriction patterns generated by *Eco*RI-digested DNA from *C. dubliniensis* hybridized poorly with the *C. albicans*- and *C. stellatoidea*-specific mid-repeat sequence probe 27A, yielding weak hybridization profiles composed of a small bands compared with the large number of strongly hybridizing band obtained with genomic DNA from *C. albicans* isolates. These findings indicated that the genomic organization of *C. dubliniensis* is significantly different to that of *C. albicans* and *C. stellatoidea* as 27A and similar probes hybridized well with repetitive DNA sequences dispersed throughout the genome of both of these species. Moreover, the authors suggested that *C. dubliniensis* might be readily distinguished from *C. albicans* on the basis of significant differences in restriction patterns generated by *Hin*f1-digested DNA in agarose gels, without the requirement for hybridizaton with probe 27A. Millon *et al* (2002) used this last technique for delineation of *Candida* isolates from HIV-infected patients. Results of RFLP analysis demonstrated that *Hin*f1 restriction of *C. albicans* produced a characteristic fragment from the variable spacer region in rDNA. The authors suggested that RFLP method generated patterns of variable complexity in agarose gels, and requested a time-consuming analysis to achieve consistent typing results. In a study by Martinez *et al* (2002), *Candida* samples were plated on CHROMagar Candida medium for initial isolation, but correct identification of the isolates as *C. dubliniensis* was only allowed by RFLP using Southern blot analysis with the species-specific probes. RFLP typing consisted of electrophoresis of *Sfi*I and *Eco*RI restriction fragments followed by Southern blot hybridization with probe Ca3 for *C. albicans*, and subsequently with probe Cd25, specific for *C. dubliniensis*. RFLP with hybridization with a *Candida*-specific probe was considered a very informative technique, but is time-consuming as blots are needed.

The ability in associate with other techniques, as amplification by polymerase chain reaction (PCR) or use of rRNA probes, seems to be the main advantage of RFLP allowing the increasing of sensitivity and applicability in clinical trials (Merz, 1990). According to Li (1997), ribosomal regions exhibit a low interspecific polymorphism, and a high interspecific variability. It has been demonstrated that restriction patterns generated from the complex ITS regions (non-coding and variable) and the 18S and 25S rRNA genes encoding regions (rDNA) are helpful in measuring close fungus genealogical relationships, including important *Candida* species such as between *C. albicans* and *C. dubliniensis* (Irobi *et al*, 1999; McCullough *et al*, 1999). Irobi *et al* (1999) used RFLPs generated from ITS of rDNA to differentiate isolates of *C. albicans*, *C. tropicalis*, *C. dubliniensis* and *C. krusei* from 114 *Candida* isolates and 65 reference strains. McCullough *et al* (1999) used two molecular methods to characterize unusual strains of *C. albicans* and to compare them with authentic strains of *C. dubliniensis* and type I *C. stellatoidea*. It was observed that the RFLPs generated by *Hae*III digestion of the PCR products of the V3 region of the 25S rRNA gene (rDNA) could differentiate the same groups as RFLP analysis of the PCR amplicon of the ITS region. The authors concluded that the method that detects the presence and the size of the intron in the 25S rDNA might be particularly easily adapted for use in reference

laboratories for the rapid identification of large numbers of isolates (including *C. albicans* and *C. dubliniensis*), with the additional advantage of differentiating strains of *C. albicans* into genotypic subgroups.

The restriction patterns generated from ITS regions together with 5.8S rRNA gene has been strongly recommended to display interspecific differences among fungus species (Williams *et al*, 1995; Esteve-Zarzoso *et al*, 1999; Graf *et al*, 2004). Williams *et al* (1995) evaluated the ITS1 and ITS2 regions, together with the entire 5.8S rRNA gene. The sequence variations in the ITS regions were amplified by PCR, using primers ITS1 and ITS4. Although PCR products from both *C. albicans* and *C. dubliniensis* had been of similar size (around 540 bp), sequence analysis revealed over 20 consistent base differences between the products of the two species. The restriction enzyme *Msp*A1 I yielded two distinct fragments from *C. albicans* PCR products at the same time as those from *C. dubliniensis* appeared undigested. The same technique was used by Gee *et al* (2002) to confirm the existence of two distinct populations within the species *C. dubliniensis*, designed Cd25 group I and Cd25 group II, respectively, on the basis of DNA fingerprints generated with *C. dubliniensis*-specific probe Cd25. More recently, Graf *et al* (2004) established a PCR/RFLP-based system with amplification of regions ITS1 and ITS2 together with the 5.8S rRNA gene, followed by digestion with HpyF10VI and separation of the DNA fragments on an agarose gel for differentiation of *C. dubliniensis* from *C. albicans*. This method provided, for all strains, PCR products of expected lengths (141, 184, and 261 bp for *C. albicans* or 264 and 325 bp for *C. dubliniensis*). In addition, it was not observed variations of the restriction patterns indicating intra-species stability of the HpyF10VI-sites used in the RFLP analysis.

Some studies have employed universal fungal primers, multicopy gene targets, and species-specific probes directed to the ITS2 region of rDNA to develop a rapid PCR assay to detect candidemia (Fujita *et al*, 1995; Salkin *et al*, 1998) in which the PCR amplicons were detected in an enzyme immunoassay (EIA) format, and the method was referred as PCR-EIA (Fujita *et al*, 1995). In a study by Elie *et al* (1998), 18 *Candida* species probes were designed and tested in the PCR-EIA against the DNAs from 35 fungal species. The PCR-EIA correctly identified multiple strains of each species tested, including *C. albicans* and *C. dubliniensis*. For these species the results showed sufficiently significant sequence differences occurred in the ITS2 region thus facilitating the development of species-specific probes. Therefore, the time to species identification by PCR-EIA after obtaining a pure culture was reduced to 7 h rather than a mean of 3.5 days by conventional techniques. According to the authors, the suggested method proved to be simple, rapid, and feasible for identifying *Candida* species in clinical laboratories, including *C. dubliniensis* and *C. albicans*. Ellepola *et al* (2003) compared phenotypic methods (i.e. the ability to grow at elevated temperatures, colony color on CHROMagar Candida medium, and carbohydrate assimilation patterns) for the differentiation of *C. dubliniensis* from *C. albicans* to amplify the results of a polymerase PCR assay using universal fungal primers to the ITS2 region of rDNA and species-specific DNA probes in a PCR-EIA. The *C. dubliniensis* ITS2 probe was specific for the identification of *C. dubliniensis* DNA and did not cross-react with DNA from any of the other *Candida* species tested (*C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, and *C. tropicalis*). The identification of *Candida* species by ITS1 rDNA sequencing gave 100% correspondence to the results obtained by the PCR-EIA, confirming the specificity of the PCR-EIA method. On the other hand, the identification of *Candida* species by ITS1 rDNA sequencing gave 100% correspondence to the results obtained by the PCR-EIA, confirming the specificity of the PCR-EIA method. The authors concluded that a combination of phenotypic methods could help to differentiate *C. dubliniensis* from *C. albicans* to some extent, but the definite identification of these two species was provided by the PCR-EIA.

Although RFLP analysis offers a reproducible approach for discrimination of *Candida* spp., the technique generally requires computer-based systems and databases for comparisons of large numbers of strains (Williams and Lewis, 2000). However, RFLP analysis obtained from *Candida* spp. may result in the generation of complex patterns that are ambiguous and difficult to interpret even by using a computer program (Millon *et al*, 2002). Therefore, practical limitations and costs related to RFLP analysis may restrict their importance for routine identifications (Williams and Lewis, 2000). To overcome these limits, RFLP method has been successfully associated with other techniques (Williams *et al*, 1995; Elie *et al*, 1998; McCullough *et al*, 1999; Ellepola *et al*, 2003; Graf *et al*, 2004).

Randomly amplified polymorphic DNA analysis or arbitrarily primed polymerase chain reaction

Random amplified polymorphic DNA (RAPD) technique, also known as arbitrarily primed PCR (AP-PCR), increased the applicability of PCR for identification of *Candida* species (Bougnoux *et al*, 1994). RAPD analysis is based on PCR amplification of target genomic DNA sequences with one or more short oligonucleotide primers (nine to 10 bases) with a low annealing temperature (36°C). RAPD products are size separated by electrophoresis processed in agarose gel. Afterwards, gels are stained with a solution containing ethidium bromide and RAPD profiles are visualized on a UV transilluminator and photographed. If a comparison of amplifications of several isolates shows a band that varies, alleles are assigned to the presence and absence of the band (Taylor *et al*, 1999). The resulting amplifications patterns are analyzed by visual interpretation or computerized programs (Soll, 2000).

Distinctive PCR profiles consisting of multiple amplified fragments were obtained by RAPD with isolates of *C. albicans*, *C. lusitaniae*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*. *C. haemulonii*, *C. glabrata* and *C. dubliniensis* (Bougnoux *et al*, 1994; Sullivan *et al*, 1995; Williams *et al*, 1995; Steffan *et al*, 1997). According to Sullivan

et al (1996), RAPD profiles of separate isolates of the same species generally showed intra-species DNA amplimer size polymorphisms. These were more similar to the patterns obtained with other isolates of the same species than RAPD profiles derived from a different species.

The first description of *C. dubliniensis* as a separate species of the genus *Candida* by phenotypic tests was further clearly established by RAPD analysis (Sullivan *et al*, 1995). Willis *et al* (2000) evaluated whether *C. dubliniensis* was carried in the oral cavity, and whether it was associated with type I diabetic. Initially, the isolates were submitted to phenotypic methods and the identification was provided by molecular methods. When genomic DNA from atypical candidal isolates was fingerprinted with the *C. albicans* 27A mid-repeat sequence probe, the isolates putatively identified as *C. dubliniensis* on the basis of carbohydrate assimilation profiles yielded fingerprint patterns that were much weaker than those obtained from *C. albicans* isolates. RAPD-PCR analysis was used to confirm these isolates as *C. dubliniensis*. Similarly, following phenotypic characterization of clinical isolates, other studies also used RAPD analysis to provide a definite identification of *C. dubliniensis* (Coleman *et al*, 1997b; Alves *et al*, 2001; Milan *et al*, 2001).

Bautista-Muñoz *et al* (2003) observed that highly consistent, clear and repetitive profiles were obtained with each of three oligonucleotide primers (OPE-18, OPE-04 and OPA-18) and collections of clinical isolates. RAPD analysis revealed differences between the profiles for *C. albicans* and *C. dubliniensis* and proved to be a reliable and repeatable technique for the identification of *Candida* species. In a study by Mariano *et al* (2003), the final identification of *C. dubliniensis* was obtained by RAPD analysis using 3 different primers (CDU, M2 and B-14). This method exhibited several high-density bands that allowed a clear visual interpretation of the DNA fingerprinting exhibited by *C. dubliniensis* and *C. albicans* isolates. Ruhnke *et al* (1999) observed that primer M13-mediated RAPD as well as with hybridization patterns using the species-specific DNA probe CARE-2 were effective to discriminate four *C. dubliniensis* strains from isolates of HIV-infected women.

Park *et al* (2000) compared a species-specific molecular beacon probe that recognizes a 22-nucleotide target region in the ITS2 region of *C. dubliniensis* with two molecular methods (RAPD analysis and restriction endonuclease analysis). The three methods showed the same results for differentiation between *C. albicans* and *C. dubliniensis*. These findings were different from those obtained by Millon *et al*, 2002 that compared three molecular methods (EK, RFLP analysis and RAPD analysis) with ITS sequences analysis. RAPD patterns of seven isolates were difficult to analyze because one of the bands produced was not of uniform intensity. Besides of computer analysis, RAPD technique often required subjective intervention of the operator to decide position, and presence or absence of a weak band. The authors suggested that RAPD analysis required some time to achieve reliable discrimination of *Candida* species, particularity *C. dubliniensis*.

From the available literature, RAPD analysis was generally described as a rapid, reliable, simple and accurate method for identification of *Candida* species, including *C. dubliniensis* (Sullivan *et al*, 1995; Coleman *et al*, 1997b; Park *et al*, 2000; Bautista-Muñoz *et al*, 2003). Furthermore, the RAPD method does not depend on prior knowledge of species-specific sequences. However, current limitations exist for routine use of the RAPD method for clinical diagnosis (Steffan *et al*, 1997). In order to preserve the reproducibility of the characteristic RAPD patterns, constant amounts of DNA must be used while other PCR methods can be optimized to directly detect smaller amounts of the target DNA present in clinical samples (Bautista-Muñoz *et al*, 2003). According to Melo *et al* (1998), RAPD analysis is not able to quantify the differences detected between isolates. The authors also stated that because of the repetitive character of the target sequences, genetic distances calculated from RAPD could be affected by paralogy, namely, recombination and duplication events not parallel with speciation events (Melo *et al*, 1998). Although nucleotide sequence information on the organisms being evaluated is not required, RAPD primers that yield informative profiles with isolates of one *Candida* spp. may not do so with another. RAPD primers that yield informative fingerprint profiles with all the *Candida* spp. isolated commonly from clinical samples have yet to be described (Sullivan *et al*, 1996).

Amplified fragment length polymorphism

Amplified fragment length polymorphism (AFLP) analysis is a relatively new technique that has been reported as an appropriate molecular tool used for identification as well as strain typing (Vos *et al*, 1995; Janssen *et al*, 1996; Savelkoul *et al*, 1999; Borst *et al*, 2003; Ball *et al*, 2004). The polymorphism exhibited by AFLP analysis depends on restriction endonuclease site differences, just like RFLPs (Vos *et al*, 1995). Nevertheless, the AFLP analysis is based on the ligation of adapters (i.e. linkers and indexers) to genomic restriction fragments followed by a PCR-based amplification with adapter specific primers (Vaneechoutte, 1996). As fragments are PCR amplified, only very small amount of DNA is needed (Savelkoul *et al*, 1999; Taylor *et al*, 1999). In the AFLP technique DNAs of any origin or complexity can be used. Fingerprints are created without prior sequence knowledge by a limited set of generic primers. The number of fragments detected in a single reaction can be 'turned' by selection of specific primer sets (Vos *et al*, 1995). Briefly, in AFLP analysis genomic DNA is digested with two restriction enzymes, one with an average cutting frequency (like *Eco*RI) and a second one with a higher cutting frequency (like *Mse*I or *Taq*I). Then, double-stranded oligonucleotide adapters are ligated to genomic restriction factors with PCR amplification of the adapter-specific primers. In this technique, the double-stranded oligonucleotide adapters are designed in such a way that the initial restriction site is not restored after ligation. Therefore, simultaneous restriction and ligation are allowed as religated fragments are cleaved again (Vos *et al*, 1995; Savelkoul *et al*,

1999). The restriction fragments analyzed are small enough for mutations of 1 bp to be detected (Janssen *et al*, 1996). To enhance the specificity, it is likely to elongate the primers at their 3′ ends with one to three selective nucleotides. The PCR primer, which spans the average-frequency restriction site is labeled. The greatest improvement has been achieved by changing from radioactive to fluorescently labeled primers for detection of fragments in a computer-based automated sequence analyzer (Vos *et al*, 1995; Savelkoul *et al*, 1999).

Borst *et al* (2003) found that AFLP patterns of the reference *Candida* strains clearly showed that each species, including *C. dubliniensis*, forms a distinct cluster. AFLP analysis demonstrated a misidentification rate of 6% in a large collection of clinical isolates previously identified on CHROMagar as *C. albicans*. Ball *et al* (2004) observed that AFLP patterns from isolates of children undergoing allogeneic stem cell showed microevolution of a *C. albicans* strain and simultaneous and transient colonization with three different species (*C. dubliniensis*, *C. lusitaniae* and *Saccharomyces cerevisiae*). AFLP method also allowed definitive discrimination of strains incorrectly identified as *C. albicans* by a rapid enzyme test.

The AFLP analysis has been considered more reproducible than RFLP genotyping as relatively small amounts of DNA are digested (Vos *et al*, 1995). In addition, the detection of AFLP fragments does not depend on hybridization and consequently partial digestion and faint patterns can easily be avoided (Savelkoul *et al*, 1999). Because of these advantages, AFLP technique may be useful for the identification and typing of medically important fungi that are difficult to cultivate (Taylor *et al*, 1999). Because stringent annealing temperatures are used during amplification, AFLP analysis has been considered more robust than other methods such as RAPD (Savelkoul *et al*, 1999). AFLP analysis of medically important *Candida* species has been considered a reliable epidemiological research tool (Vos *et al*, 1995; Savelkoul *et al*, 1999; Ball *et al*, 2004). Furthermore, AFLP method has been recommended as suitable molecular technique for clinical use that might provide the identification of medically important *Candida* species, including *C. dubliniensis* and *C. albicans* (Borst *et al*, 2003; Ball *et al*, 2004). However, as AFLP analysis depends on expensive computer software, the patterns obtained are not easy to exchange between different laboratories (Ball *et al*, 2004).

Mini- and microsatellites

Many organisms, both prokaryotic and eukaryotic, contain highly repetitive short DNA sequences throughout their genomes. Depending on the rate of evolution, these sequences may be detected and allow differentiation to the species or strain level (Zeze *et al*, 1996). The class of repetitive DNA named variable number of tandem repeats (VNTR) can consist of several hundreds to several thousands of base pairs of DNA in head-to-tail repetition of short sequence motifs (Jeffreys *et al*, 1985). Micro- and minisatellites are defined as short tandem repeats (STRs) of two to six and six to 100 nucleotides, respectively (Bruford and Wayne, 1993). They are excellent sources of polymorphism, as the number of repeat units can increase or decrease because of slippage of the DNA strands during replication by the DNA polymerase (Bruford and Wayne, 1993; Metzgar *et al*, 1998a; Kirk *et al*, 2004). The rationale for STR approach was that microsatellite regions are codominantly inherited and allow the distinction of heterozygotes, which is critical in the case of the diploid yeasts such as *C. albicans* and *C. dubliniensis* (Whelan and Magee, 1981). STRs have been used as molecular markers in fields so diverse as oncogenetics, population structure studies and genetic mapping (Bruford and Wayne, 1993; Metzgar *et al*, 1998a; Sampaio *et al*, 2003; Kirk *et al*, 2004). In addition, these markers have been found to be useful for molecular typing as length polymorphisms are often detected between isolates within the same species. The polymorphism of the STRs can be analyzed after amplification of specific loci by use of fluorescent primers and migration in a high-resolution gel (Bretagne *et al*, 1997). The molecular mechanism for producing differences in allele sizes is primarily because of polymerase slippage errors (Strand *et al*, 1994). Reliability can be achieved by an automated procedure, i.e. an automatic sequencer is used to measure the length of the alleles (Hunter, 1991).

By using short oligonucleotide probes complementary to STR microsatellites sequences, including $(GGAT)_4$, $(GACA)_4$, $(GATA)_4$, $(GTG)_5$, and $(GT)_8$, informative DNA fingerprint profiles consisting of multiple hybridization bands – ranging in size from approximately100 bp to 20 kb for isolates of *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. krusei*, *C. dubliniensis* and *C. stellatoidea* – can be obtained (Sullivan *et al*, 1995; Diaz-Guerra *et al*, 1999; Meyer *et al*, 2001; Sampaio *et al*, 2003). However, few studies have applied STR-based methods with the objective of differentiating *C. dubliniensis* from *C. albicans*. Meyer *et al* (2001) used the microsatellite $(GACA)_4$ and the minisatellite (5′GAGGGTGGCGGTTCT-3′), derived from the core-sequence of the wild-type phage M13 specific oligonucleotides as single primers in PCR to amplify hypervariable inter-repeat DNA sequences from 16 *C. dubliniensis* and 11 *C. albicans* strains. Each species could be identified by a distinct species-specific multilocus pattern, allowing identification to species level for all clinical isolates. Minisatellite specific primer M13 was more discriminatory than the $(GACA)_4$. The authors recommended PCR-fingerprinting using specially the primer M13 as a simple, rapid and reproducible molecular tool to distinguish *C. dubliniensis* and *C. albicans*. Diaz-Guerra *et al* (1999) used PCR-fingerprinting with the core sequence of phage M13 to confirm the identity of six *C. dubliniensis* isolates recovered from two HIV-infected individuals in the course of a prospective study of recurrent oral candidosis. The fingerprinting profiles of *C. dubliniensis* isolates were distinguishable from those seen for *C. albicans* strains. The PCR-profiles of the *C. dubliniensis* isolates lacked a highly conserved band of about 2.0 kb that has been observed in all *C. albicans* patterns obtained with the

M13 primer. The amplification patterns obtained with the core sequence of the phage M13 exhibited an easily analyzable number of bands, amenable to computerized analysis. In addition, PCR patterns obtained with this method exhibited band polymorphisms between strains from different patients and even between strains from a single patient. Although few strains were analyzed, the PCR-fingerprinting procedure was considered as a promising tool for further epidemiologic studies with *C. dubliniensis*. On the contrary, Bartie *et al* (2001) found that inter-repeat PCR methods (primers 1245 and 1246 and primer 1251) were more reproducible than M13 primer to assess the genetic relatedness of *C. albicans* isolates from an epidemiologic analysis of the of chronic hyperplastic candidosis. The primer 1251 was found to be the most effective primer for the detection of DNA polymorphism between strains of *C. albicans*. Variations in the intensities of fragments amplified with the M13 primer, particularly with the low-intensity bands, could have compromised the reproducibility of the method. Eight isolates that were initially identified as *C. albicans* by the API 32C system were later confirmed as *C. dubliniensis* by inter-repeat PCR.

Metzgar *et al* (1998a) demonstrated that ERK1 locus and several other polymorphic microsatellite loci could act as species-specific markers. Of seven loci analyzed in *C. albicans*, only one (ERK1) could be amplified from *C. dubliniensis*, and none of them could be amplified from *C. krusei*. The use of these markers in strain identification was restricted to *C. albicans* although the identification of a polymorphic ERK1 homologue in *C. dubliniensis* suggested that specific design could provide microsatellite based strain identification systems for species of yeast other than *C. albicans*. To determine the potential of compound microsatellites as informative fungal genetic markers and to resolve length homoplasies, the authors further sequenced 21 alleles of ERK1 from 14 samples of *C. albicans* and, for interspecies comparison, three alleles from two samples of *C. dubliniensis*. The ERK1 locus was length variable in *C. dubliniensis*, although many of the variable regions seen in *C. albicans* do not have homologous repeats in *C. dubliniensis*. They concluded that microsatellite length alone should not be used to assume either sequence identity or identity by descent.

To date, most of polymorphic microsatellite loci described in *C. albicans* was located near or inside coding regions. The discriminatory power calculated for these STRs was between 0.77 and 0.91 (Bretagne *et al*, 1997; Metzgar *et al*, 1998b). Based on these findings, Sampaio *et al* (2003) investigated the polymorphism of a new microsatellite locus (CAI), outside a known coding region, in the genome of *C. albicans* strains. The microsatellite was considered stable with a discriminatory power of 0.97. Further studies are needed to evaluate the polymorphism of microsatellite locus, outside coding regions, in *C. dubliniensis* strains.

Although the mini- and microsatellites markers are stable over many generations (Stéphan *et al*, 2002), information may be lost through marker mutation as microbes have short generation times (Bretagne *et al*, 1997). The use of STRs to study microbial diversity may be limited depending on the complexity of the community (Kirk *et al*, 2004). Furthermore, variability because of a high frequency of change in satellite DNA sequences may decrease the effectiveness of the method in clustering moderately related isolates (Metzgar *et al*, 1998a). Another limitation of these methods is that the sequences of the mini- and microsatellite regions need to be known so appropriate primers can be used (Kirk *et al*, 2004). On the contrary, it has been demonstrated that STR-PCR-based methods have several advantages over the other methodologies used in strain identification, as mini- and microsatellites are known to be highly polymorphic and the PCR is a less time-consuming technique (Metzgar *et al*, 1998b). Moreover, PCR typing mini- and microsatellites may result in a higher level of reproducibility than RAPD because the annealing temperature is higher (Diaz-Guerra *et al*, 1999; Soll, 2000). These techniques may fulfill several of the biological and technical criteria, such as high polymorphism, reproducibility, and feasibility that denote reliability for use in epidemiological studies (Hunter, 1991). The digitization of the data permits the fingerprinting method to be portable and comparable among laboratories. In addition, if appropriate primers are used, STR-PCR-based methods may be used to provide a definitive discrimination between *C. dubliniensis* and *C. albicans*.

Future directions of molecular procedures for the identification between C. albicans and C. dubliniensis

Peptide nucleic acid (PNA) probes are DNA probe mimics with a polyamide backbone to which individual nucleobases are attached. This structure provides the PNA probes with improved hybridization characteristics such as high degrees of specificity, strong affinities, and rapid binding kinetics, as well as an enhanced ability to hybridize to highly structured targets (Egholm *et al*, 1993). In particular, the relative hydrophobic character of PNA allows PNA probes to diffuse through the cell wall under conditions, which do not lead to the disruption of cell morphology (Stender *et al*, 1999). PNA FISH is a new technique based on fluorescent *in situ* hybridization (FISH) assays that combines the performance characteristics of PNA probes with the use of rRNA as a target (Egholm *et al*, 1993; Stender *et al*, 1999). Oliveira *et al* (2001) designed specific PNA probes targeting the rRNAs of *C. albicans* and *C. dubliniensis* and applied them to a PNA FISH method for differentiation between these two species. The results showed that PNA FISH method using smears of 79 *C. dubliniensis* and 70 *C. albicans* strains showed 100% sensitivity and 100% specificity for both PNA probes. The authors concluded that PNA FISH is a powerful tool for the differentiation of *C. albicans* and *C. dubliniensis*. Rigby *et al* (2002) designed a PNA probe that targets *C. albicans* 26S rRNA and used PNA FISH for the identification of *C. albicans* directly from blood cultures. The specificity of the method was confirmed with 23 reference strains and 148 clinical isolates covering the clinically most significant yeast species,

including 72 isolates of *C. albicans* and 48 isolates of *C. dubliniensis*. According to the authors, PNA FISH method for the definitive identification of *C. albicans* directly from yeast-positive blood culture bottles provided important information for optimal antifungal therapy and patient management.

The reverse hybridization line probe technology (LiPA) is based on the reverse hybridization principle: biotinylated PCR fragments are hybridized to a selection of highly specific immobilized probes. In a second step, the biotin group in the hybridization complex is revealed by incubation with a streptavidin-alkaline phosphatase complex and the appropriate chromogen compounds (White *et al*, 1990). In a study Martin *et al* (2000), species-specific oligonucleotide probes were designed from within the ITS region for several fungal species. These probes were incorporated into a LiPA, combined with PCR amplification of the ITS region, and evaluated on a panel of typed fungi and clinical isolates. Sequence analysis of the ITS regions from both *C. albicans* and *C. dubliniensis* revealed a high degree of homology between both species. It was observed cross-reaction between the ITS PCR products from these two species and their respective oligonucleotide probes. The CD2 probe, which was specific for *C. dubliniensis*, was designed from the ITS2 region, and a second probe, CD3, designed from ITS1 with a single-base-pair mismatch to *C. albicans* in the ITS1 probe region, was also specific for detection of *C. dubliniensis*. LiPA was recommended as robust and sensitive technique for discrimination of a small number of clinical fungal isolates.

The use of standardized DNA extraction protocols and real-time PCR may address many of the limitations of conventional PCR. More recently, a quantitative real-time PCR assay using the Light-Cycler (Roche Molecular Diagnostics, Mannheim, Germany) and Taq-Man (Perkin-Elmer Corp., Applied Biosystems, Foster City, CA, USA) instruments have been reported to show great potential for the rapid *in vitro* amplification and detection of *Candida* species (White *et al*, 2004). The 5′ exonuclease (TaqMan^{TM}) assay is a homogenous system using a fluorescent labeled probe for the detection of PCR products. The primer probe sets can be used individually or combined in two multiplex sets. According to Guiver *et al* (2001), this method provides a rapid automated combined PCR amplification and detection system with no postamplification manipulation of amplicons, thus significantly reducing the risk of contamination. Coupled with a quick, simple DNA extraction method, this protocol may permit rapid discrimination of clinical isolates. Recently, a Taq-Man^{TM} PCR was evaluated for the rapid identification of clinically important *Candida* species. The primer and probe sets have been shown to be 100% specific. The presence of two putative *C. dubliniensis* clinical isolates originally identified as *C. albicans* on the basis of germ-tube formation and chlamydospore production was highlighted by the absence of amplification with the *C. albicans* primer and probe set. The authors concluded that the simple extraction method followed by Taq-Man^{TM} PCR can identify important *Candida* species in 4 h. Alternatively, Real-time PCR technology with the Light-Cycler was recently developed for the sensitive and rapid (1 day) detection of *Candida* species, including *C. albicans* and *C. dubliniensis* (White *et al*, 2004). Although the costs of PCR generally exceed those of conventional culture methods and may currently limit its widespread use, earlier and more sensitive diagnosis could eventually decrease the high mortality of fungal infections in immunocompromised patients (Imhof *et al*, 2003).

Other methods for discrimination between C. albicans and C. dubliniensis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis

The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) has emerged as one of the most versatile and inexpensive techniques for the separation and resolution of bacterial proteins that may be used for the establishment of the phenotypic variability and relationship within species (Kersters, 1985). Many studies have been used this technique for the identification of important strains (Cunningham and Noble, 1989; Rosa *et al*, 2000; Boriollo *et al*, 2003; Rosa *et al*, 2003; Rodrigues *et al*, 2004). The comparison of electrophoretic protein patterns has satisfactory taxonomic resolution, which may be applicable to the level of species, subspecies and biotypes (Kersters, 1985). SDS-PAGE protein profiles are generally obtained after electrophoresis of denatured protein solutions in polyacrylamide gel. The images of the gels are captured using a scanner and the relative mobility of each protein band is determined with specific software. Dendrograms are generated by specialized computer programs after the overall gel analysis (Kersters, 1985).

While various studies have recommended the SDS-PAGE as a useful technique for discriminating different *Candida* species, particularly *C. albicans* (Rosa *et al*, 2000; Boriollo *et al*, 2003; Rodrigues *et al*, 2004), little information is available on pertinent literature concerning the use of this technique for identification of *C. dubliniensis*. Multilocus enzyme electrophoresis (MLEE) and SDS-PAGE were recently used to separate *C. albicans* and *C. dubliniensis* into different taxa (Rosa *et al*, 2003). The results showed that MLEE could separate both species with a smaller similarity than SDS-PAGE, suggesting that SDS-PAGE generated a greater number of bands, most of them common to *C. albicans* and *C. dubliniensis*. The authors concluded that, when all the data were analyzed together, MLEE and SDS-PAGE could be recommended as consistent methods for discrimination between the two species.

Because of its low costs, reproducibility, convenience, simplicity and possibility of reducing the number of isolates that would be analyzed by other molecular methods more complex, SDS-PAGE analysis has been investigated for identification of clinically significant strains (Rosa *et al*, 2000; Boriollo *et al*, 2003; Rosa *et al*, 2003). However, the use of this technique as *Candida* species discriminatory tool is still controversial (Cunningham and Noble, 1989). Complementary studies

must be carried out to evaluate the efficacy of SDS-PAGE analysis for identification between *C. albicans* and *C. dubliniensis*.

Multilocus enzyme electrophoresis

Multilocus enzyme electrophoresis is a protein based typing method whose results can be directly correlated with the genotype (Wang *et al*, 1999). The MLEE technique has been used to evaluate genetic diversity, gene flow, and population or genotypic structure, besides being valuable for typing, systematic, and epidemiological studies of medically important fungi (Taylor *et al*, 1999; Soll, 2000). MLEE evaluates the polymorphism of isoenzymes or allozymes of the isolates. In MLEE technique, proteins from cell extracts are separated by starch gel electrophoresis, polyacrylamide gel electrophoresis, or isoelectric focusing under native (non-denaturing) conditions and the electrophoretic mobility of metabolic enzymes is determined after specific enzyme-staining procedures (Taylor *et al*, 1999; Wang *et al*, 1999; Soll, 2000). Each electromorph is equated with an allele at the corresponding enzyme genetic locus. Therefore, by associating each isolate with an electrophoretic type, MLEE allows the differentiation of isolates by marking them with significant characteristics (Wang *et al*, 1999). To obtain complex data for computing a similarity coefficient between two isolates, several enzymes must be assessed (Soll, 2000).

Although there are several studies based on MLEE (Boerlin *et al*, 1996; Pujol *et al*, 1997) describing intraspecific diversity, population structure, and mode of reproduction of *Candida* species, little information could be found regarding the interspecies discriminatory power of MLEE technique for *C. albicans* and *C. dubliniensis*. Boerlin *et al* (1995) used MLEE technique and hybridization with the *C. albicans*-specific Ca3 probe to examine chlamydospore-forming and germ-tube positive *C. albicans* clinical isolates of human immunodeficiency virus HIV-positive and found two main groups of strains. One group contained strains with atypical sugar assimilation patterns and could be distinguished from the other group by the absence of intracellular β-glucosidase activity. This group was later confirmed as being *C. dubliniensis* (Sullivan *et al*, 1999). Using several phenotypic methods and MLEE technique, Badoc *et al* (2002) typed strains of atypical *C. albicans* isolated in laboratory and compared them to reference strains of *C. dubliniensis* and strains of *C. albicans* isolated from HIV-positive patients. MLEE analysis showed an extensive variability of *C. albicans* and this diversity was consistent with the large number of enzymes tested and the extensive heterogeneity of strains studied. According to the authors, MLEE could distinguish *C. dubliniensis* and the atypical *C. albicans* strains from all strains of *C. albicans*. Furthermore, this technique could identify atypical *C. albicans* strains as *C. dubliniensis*. The results of a study by Rosa *et al* (2003), obtained using a single buffer system and a small number of enzymatic revelations, generated a MLEE dendrogram in which *C. dubliniensis* could be separated from *C. albicans* in an efficient manner.

The MLEE method has been considered straightforward and good for studies of medically important fungi (Taylor *et al*, 1999; Wang *et al*, 1999; Soll, 2000). Furthermore, this method has been recently suggested as a robust typing tool to discriminate among isolates of *C. dubliniensis* and *C. albicans* (Badoc *et al*, 2002; Rosa *et al*, 2003). However, the major drawback to MLEE method is that it is relatively time-consuming, because one must combine the data from at least 10 enzymes that provide variability among isolates (Pujol *et al*, 1997). In addition, in MLEE technique, the genotype is evaluated indirectly. Then, variation at the nucleotide level may go undetected because nucleotide substitutions do not essentially change the amino acid composition (Karl and Avise, 1992; Pogson *et al*, 1995). Moreover, changes in amino acid composition do not necessarily change the electrophoretic mobility of the protein, and, as a result, alleles considered to be homologous from different individuals may represent different gene alleles (Karl and Avise, 1992; Pogson *et al*, 1995). Second, selection may be acting on the polymorphisms, so that anonymous DNA markers may give a very different picture from allozyme markers, presumably because the former are neutral and the latter are under some sort of selection (Karl and Avise, 1992; Pogson *et al*, 1995; Taylor *et al*, 1999).

Conclusions

Although *C. albicans* and *C. dubliniensis* share many phenotypic characteristics, they may differ with respect to its epidemiology, virulence, and the ability to develop fluconazole resistance. Therefore, the identification between these two species is crucial for clinical treatment and epidemiological studies. Most of molecular fingerprinting techniques have been considered extremely sensitive and precise to provide definitive identification between *C. albicans* and *C. dubliniensis*. However, the use of these methods in routine diagnostic laboratories may be limited because many of them have yet some drawbacks such as relatively labor intensive, costly, time-consuming and difficult to apply to large numbers of isolates. To date, molecular methods are more suitable for specialized laboratories such as research or regional diagnostic labs. Consequently, a rapid, simple and cost-effective molecular fingerprinting method for the discrimination of these species needs to be investigated for more timely and properly disease management especially in clinical laboratories receiving large volumes of yeast isolates from immunocompromised patients. Moreover, the ideal DNA fingerprinting method should produce invariable results from laboratory to laboratory to permit unequivocal comparative analyses and the establishment of reliable databases.

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