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# Molecular-Genetic Approaches for Identification and Typing of Pathogenic Candida Yeasts: A Review

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**ABSTRACT**: Candida is a medically important fungi because of its high frequency as a commensal and pathogenic microorganism causing superficial as well as invasive infections. Because the accurate diagnosis of candidiasis remains difficult, a fast and reliable assay for characterization of fungal pathogens is critical for the early initiation of adequate antifungal therapy and/or for introduction of preventive measures. As novel molecular genetic techniques are continuously introduced, their role in the management of infectious diseases has also been growing .Strain typing and delineation of the species are essential for understanding its biology, epidemiology and population structure. A wide range of molecular techniques have been used for this purpose including non-DNA-based methods (multi-locus enzyme electrophoresis), conventional DNA-based methods (electrophoretic karyotyping, random amplified polymorphic DNA, amplified fragment length polymorphism, restriction enzyme analysis with and without hybridization, rep-PCR) and DNA-based methods called exact typing (PCR based methods, microstallite length polymorphism, multilocus sequence typing, DNA microarray ) because they generate unambiguous and highly reproducible typing data. Today, molecular strategies complement conventional methods and provide more accurate and detailed insight. It can be expected that future technical development will improve their potential furthermore.In this article, we provide a critical review on the vigorously fermenting field of molecular approaches to *Candida* identification and typing and summarized their advantages and limitations with regard to their discriminatory power, reproducibility, cost and ease of performance.

**KEYWORDS**: *Candida* , sequence polymorphisms, molecular approaches, strain typing, genetic diversity,

## I. INTRODUCTION

Candida is a common commensal microorganism in humans and some warm blood animals. The standard genome of C. albicans is diploid and composed of eight pairs of chromosome homologs, ranging in size from about 33 to 095 Mb [1]. Candida albicans has a predominantly clonal mode of reproduction.It was the first medically important pathogenic fungus for which the complete genome sequence was determined [2]. The first genome to be sequenced was that of the SC5314 clinical isolate that showed a high degree of heterozygosity, including more than 55 700 single-nucleotide polymorphisms (SNPs) in the 32-Mb diploid genome [2]. Despite its diploidy and clonal reproduction, C. albicans is able to achieve a high rate of a genetic diversity in several ways, including recombination, chromosomal polymorphisms, gene replacement and cryptic mating, reflecting the plasticity of the genome [1],[3].Over the last three decades, C. albicans has become a medically important pathogen, responsible for superficial as well as deep infections. Most infections are opportunistic depending on the immune defence of the host and changes of the environment of the yeast in the organism.Indeed, since the early 1980s, invasive candidiasis, mainly caused by Candida species, has emerged as a prominent problem [4], because of the increasing number of immunocompromised patients and the advances in medical intensive care.

This underlines the need for implementing appropriate control measures, which obviously requires a high knowledge of the biology and the epidemiology of Candida species including C. albicans, that are recognized to be particularly complex. Indeed, the same healthy individual can harbour the same strain at different body locations, or carry unrelated strains at the same or different body sites [5] , strains can replace each other in recurrent infections [6] , undergo microevolution (minor changes in genotype over a relatively small number of cell generations [7] and substrain

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shuffling (changes in subpopulations within individuals over time) be transferred from one individual to another [8],[9],[10], specific strains may predominate in particular geographical areas and some strains may be endemic in some hospitals and can undergo microevolution in the hospital setting.This highlights the need for efficient methods for typing and delineation of strains. The earliest methods used in the typing of C. albicans and non albicans were based on phenotypic characteristics including serotyping, biotyping, morphotyping, resistance to various chemicals and toxins and antifungal susceptibility profiles [11]. However, phenotypic techniques have a very low degree of discrimination and reproducibility, which obviously constitutes a limitation for reliable epidemiological analysis. The advent of the molecular DNA-based techniques revolutionized the knowledge on the biology and epidemiology of C. albicans. The review aims (i) to describe the principles of these molecular approaches, (ii) to highlight their strengths and their drawbacks and (iii) to discuss their input in different types of C. albicans molecular investigations.

## II. RELATED WORK

Review papers on the molecular biology of *C. albicans* are available [7], [12], [13], [14], [15] which address the current advances made in the understanding of *C. albicans* molecular genetics. In addition, a very useful WWW Server on *C. albicans* research (http://alces.med.umn.edu/candida.html) has been created by Dr. Scherer (Univ. Minnesota, St. Paul, MN, USA) in which information on genetics, physical mapping, sequence data and other useful resources (methodology, metabolism, morphology, etc.) about this organism are included.[16]. Compared to blood cultures and phenotypic methods, DNA based techniques have been adopted by several microbiological laboratories for rapid and objective identification of *C. albicans*. The sensitivity of a DNA based assay is dependent on sample preparation, primer and DNA target selection, extraction of DNA, and amplification effectiveness Tsui et.al [17] detected and identify many species of fungi *Clavariopsis aquatic, Tetracladium marchalianum in plant by FISH; Phyllosticta, Coleophoma, Epicoccum, Godronia, Alternaria, Pestalotia,* and *Pilidium* in animals by DNA microarray; different species of *Candida, Cryptococcus neoformans* complex, *Saccharomyces cerevisiae,Fusarium, F. solani,* and *Scedosporium prolificans* in human by MT PCR method. Likewise Spampinato et al [18] used multilocus sequence typing (MLST) and microsatellite length polymorphisms (MLP) analysis that relies on DNA sequences of internal regions of various independent housekeeping genes, and typed *C.albicans* and C.non *albicans* species and tells us that both methods generate unambiguous and highly reproducible data. Among several techniques, RAPD and RFLP and EK were the most widely used techniques by many scientists [19], [20], [21], [22], [23] for typing of *C. albicans*, other *Candida* species (e.g. *C. dubliniensis, C. parapsilosis, C. lusitaniae, C. tropicalis, C. glabrata and C. krusei)* and other fungi (e.g. *Aspergillus fumigatus, Aspergillus flavus, Cryptococcus neoformans and Blastomyces dermatitidis*) Skrzypek et .al [24] in his paper sequence the genomes of other less infectious *Candida* strains, and the complete genome sequences for *C. albicans* (WO-1), *C. dubliniensis, C. tropicalis, C. parapsilosis, Candida guilliermondii,* and *Candida lusitaniae* are available now. The *Candida* Genome Database (CGD) is constantly updating and several of them are widely available to the research community (e.g., CandidaDB at http://genodb.pasteur.fr/cgi-bin/WebObjects; Genolevures at http://www.genolevures.org/yeastgenomes .html Sanger Institute at http://www.sagar.ac.uk/projects/ Fungi/ and Broad Fungal Genome Initiative at http://www. broad.mit.edu/node/304).This has undoubtedly opened enormous avenues to have comparative study of the genomes to identify the potential candidate genes responsible for virulence.[25].PCR based analysis offer a number of technical advantages over conventional typing methods including extremely high DP values and reproducibility, ease of use, and rapid reliable data.The selection of the technique depends on the purpose of the study, the accessibility of genotypic strains archives, the time available to complete the analysis, and the cost. Guiver et al [26] used TaqMan PCR for rapid identification of clinically important *Candida* species (*C. albicans, C. glabrata, C. tropicalis, C. krusei, C. parapsilosis, and C. kefyr*), and primers and probe sets have shown 100% of specificity for their respective species. Likewise, Selvarangan et al [27] observed that six Candida spp. (*C. albicans, C. glabrata, C. parapsilosis, C. tropicalis, C. krusei, andC. lusitaniae*) targeted by the real-time PCR approach using the LightCycler software were identified with 100% sensitivity and 100% specificity. Bougnoux et al [28] reported sensitive rolling-circle amplification (RCA)-based method using real-time PCR and Nested PCR for identification of clinically important *Candida, Aspergillus, and Scedosporium species.* The development of DNA sequence-based technologies led to a great progress in understanding the epidemiology of clinical isolates of *Candida* species.Here, in the review we focused to address a broad range of molecular techniques that have been used for identification and typing Candida species in detail.

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**GENERAL CONSIDERATIONS**

Genotyping of Candida strains allows the following: (i) investigation of nosocomial candidiasis to identify outbreak-related strains, distinguish epidemic from endemic or sporadic strains and determine the origins of infection, the routes of acquisition and transmission of strains; (ii) assessing the diversity of isolates within a carrier and investigation of the recurrent infections to recognize particularly virulent strains if any; (iii) monitoring of the emergence of drug-resistant strains; (iv) studying of the population structure, diversity and dynamics of the species. The choice of the method for strain typing is contingent upon the nature and the defined objectives of each molecular investigation. The performance of each typing technique should be assessed in terms of discriminatory power, reproducibility and ease of performance and interpretation. Discriminatory power refers to the ability of the technique to identify the same strain or highly related strains in independent isolates, to assess microevolution within the same strain, to group or cluster moderately related strains and to discriminate between unrelated strains [29],[30].

## III METHODS FOR IDENTIFICATION OF *CANDIDA* SPECIES

**A) Non-DNA-Based Typing Methods: 1.Multi-locus enzyme electrophoresis (MLEE)**

MLEE or isoenzyme typing characterizes enzymatic proteins and assesses their polymorphism by analysing their electrophoretic mobility on gels after specific enzyme staining procedures. The migration of an enzyme is influenced by its molecular size and its net charge. Changes in the mobility of an enzyme protein reflect a change in its amino acid sequence and, thus, by inference, the encoding DNA sequence. Therefore, if the enzyme banding patterns of two isolates differ, such differences are assumed to be DNA based and heritable. MLEE is a reliable technique with a relatively high discriminatory power in the distinction between unrelated strains and shows good reproducibility [30],[31].In the field of medical mycology, isoenzyme typing has shown great potential in studies of taxonomic, systematics, genetics, evolutive, and epidemiologic characterization, especially for the yeast C. albicans [32]. An enzyme reaction can be demonstrated by electrophoretic bands of enzymatic activity in gels, indicating the existence of isoenzymes or isozymes. Isoenzymes constitute multiple molecular structures of the same enzyme with individual affinity for the same substrate, catalyzing the same cellular reaction . Its control occurs genetically through one or several alleles or genes, situated at one or several loci . Isoenzymes controlled through alleles from a single locus are known as alloenzymes or allozymes .Its electrophoretic migration is determined by its net electrical charge based on amino acid sequence, and consequently, mobile variants (electromorphs or alloenzymes) can be directly compared with alleles of the corresponding structural gene locus . For *N meningitidis* serogroup A, MEE has been used, in conjunction with outer membrane protein profiles, to divide 423 strains into four major families composed of 21 clones. Subsequently, MEE was used to detect the intercontinental spread of group A disease due to a clone (designated III-1) which caused outbreaks in Nepal, Saudi Arabia and Chad [33] It outperforms many of the DNA-based methods and remains a useful tool for molecular investigation of natural populations of C. albicans [34]. The major drawback of the MLEE is that it assays the genome indirectly and evaluates variations accumulated very slowly in the species. On the other hand, MLEE does not detect all variations at the nucleotide level as nucleotide substitutions do not necessarily lead to a change in the amino acid composition of the enzyme.

**B) Conventional DNA-Based Typing Methods**

**1.Pulsed-field gel electrophoresis (PEGE): *Electrophoretic karyotyping***

The advent of pulsed-field gel electrophoresis (PFGE) technique in 1984 revolutionized the study of the genome organization of eukaryotic organisms. In this technique and its variants [orthogonal-field alternative gel electrophoresis (OFAGE), field inversion gel electrophoresis (FIGE), contourclamped homogeneous electric field (CHEF) or transverse alternate field electrophoresis (TAFE)], intact DNA molecules migrate through an agarose gel matrix under the influence of pulsed fields, which permit easy separation of DNA molecules of several megabases. As the size of C. albicans chromosomes range from around 1 to 4 Mb, this technique is ideal for the separation of chromosomesized DNA molecules, the analysis of the chromosomal banding patterns, known as electrophoretic karyotypes, and the detection of karyotypic variations within the Species.The earliest use of electrophoretic karyotyping (EK) demonstrated the extent of variation in the karyotypes of unrelated C. albicans isolates [35], [36]. Indeed, despite the occurrence of a standard karyotype that consists of eight pairs of homologous chromosomes, variant karyotypes are very common among clinical isolates. Differences in the number of bands detected and their mobility patterns were presumed to be

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due to chromosome-length polymorphisms (different-sized chromosome homologs), reciprocal chromosome translocation or missing chromosomes [37]. EK has been extensively used to fingerprint C. albicans and other Candida species (e.g. C. lusitaniae, C. parapsilosis, C. tropicalis and C. glabrata) [14], [19]. It has a moderate discriminatory power when used for typing unrelated C. albicans isolates and does not allow the discrimination between moderately related isolates nor the detection of microevolutionary changes in the same strain. Due to the excellent reproducibility, electrophoretic karyotyping has been employed extensively as a molecular marker for C. albicans .This method showed greater discriminatory power compared to RFLP without hybridization, in assays of independent isolates.However, EK shows good reproducibility, and the interpretation of the karyotypes is straightforward [36].

**2. Restriction enzyme analysis (REA)**

Restriction enzyme analysis (REA) was widely used in the earliest epidemiological investigations of C. albicans infections [38],[39],[40],[41].In this technique, total genomic DNA is purified and subsequently cleaved by a frequent cutting restriction endonuclease (e.g. EcoRI, MspI, BglII, HinF1 or HindIII) that produces a large number of short fragments resulting in a sequence-dependent restriction fragment length polymorphism (RFLP).The generated fragments are separated using common agarose gel electrophoresis and visualized after staining with ethidium bromide. Variation between strains is evidenced by different banding patterns. These variations occur as a result of changes or deletion of restriction site sequences or DNA deletions and insertions between the recognition sites .REA is straightforward, rapid and inexpensive. However, it may result in the generation of complex patterns with a large number of bands of unequal intensities, thereby making their objective interpretation and the differentiation of strains very difficult, whether visually or using computer-assisted methods.To simplify the banding patterns obtained by restriction endonuclease digestion and to increase the discriminative power of REA analysis, generated fragments can be hybridized with species-specific radiolabelled or biotinylated DNA probes after transfer to nitrocellulose or nylon membranes (Southern blotting). Probes recognize repetitive sequences dispersed throughout the genome as a result of sequence homology. In contrast to ethidium bromide staining, hybridization probes allow the selective visualization of a limited number of fragments and generate profiles that are easier to interpret but, at the same time, complex enough to provide an accurate and sensitive measure that reflects the relatedness of isolates [42]. The most commonly used probes are the related (but nonidentical) ,27A and Ca3 probes containing repetitive genomic sequences .These probes contain sequences of RPS repetitive elements and non-RPS sequences of C. albicans , which consequently hybridize with a large part of the same bands in Southern blot hybridization assays . However, these two probes are not identical. The 27A probe contain sequences downstream from the RPS groups that hybridize with single bands, while the Ca3 probe contains sequences upstream from the RPS groups that hybridize with single bands [43]. For comparison, the Ca3 probe shows greater complexity than does 27A, and moreover, contains an additional repetitive sequence called sequence B. In general, its pattern also shows more complexity and satisfies the four requirement necessary for an effective DNA fingerprinting method .The Ca3 and 27A probes were also employed in various epidemiologic studies of C. albicans and the phylogenetically related species C. dubliniensis .When used to probe Southern blots of EcoRI-digested DNA, Ca3 identifies over 20 bands of various intensities that include invariable (monomorphic), moderately variable and hypervariable bands [44]. The Ca3 probe has been demonstrated to be highly effective in the analysis of C. albicans populations and has identified five major clades (named I, II, III, SA and E) of closely related strain types [45],[46].In addition, Southern blot hybridization with probe 27A also shows greater discriminatory power in clinical strains of C. albicans when compared to results from EK, REA with Not I and PCR fingerprinting .Inherent drawbacks of Southern hybridization techniques include several laborious and time-consuming steps and the need to use DNA probes, which requires a transfer procedure to a solid support as well as adequate detection systems making implementation of the method in a medical laboratory analysis difficult. In addition, fingerprint data do not lend themselves to inter laboratory data exchange.

**3.Random amplified polymorphic DNA (RAPD)**

The RAPD technique is based on the amplification of genomic DNA with single short (typically 10 bp) primers of arbitrary sequence. Primers bind at random to the target DNA resulting in the amplification of fragments of unknown sequence. The amplification reaction is carried out under conditions of low stringency (typically 35–40°C, 25 mmol l MgCl2). Amplified products are separated on an agarose gel and stained with ethidium bromide. The interpretation of RAPD patterns is based on the number and the size of the amplified fragments. Overall, the RAPD assay generates

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relatively complex patterns that greatly vary among unrelated isolates. RAPD has been extensively used for typing of C. albicans, other Candida species (e.g. C. dubliniensis, C. parapsilosis, C. lusitaniae,C. tropicalis, C. glabrata and C. krusei) [20],[21] and other fungi (e.g. Aspergillus fumigatus, Aspergillus flavus, Cryptococcus neoformans and Blastomyces dermatitidis) [22],[23] because it offers many advantages including simplicity, rapidity and cost-effectiveness and does not require any DNA sequence information [20]. The discriminatory power of RAPD is moderate; nevertheless, if a greater level of discrimination is required, different primers can be used in independent runs and the data combined for the final analysis (composite DNA type).This approach allows the discrimination between unrelatedisolates, clustering of related isolates, identification of dentical or highly related isolates and assessment of microevolution within the same strain. Indeed, Pujol et al. demonstrated a high concordance between RAPD analysis, MLEE and Southern blotting using the Ca3 probe. The Ca3 probe exhibited the greatest resolving power mainly in assessing microevolution within the strain [47]. Although the RAPD technique has been used successfully in several clinical studies, it raises the well-known problem of reproducibility and data comparison between laboratories. Indeed, banding patterns are easily affected by slight changes in the experimental conditions (Mg2+ concentration, primer-to-template–concentration ratio,Taq polymerase concentration and source, the model of thermal cycler, thermal cycling parameters, etc.) [48].Nevertheless, a good interassay reproducibility can be achieved under rigidly controlled conditions.

**4. Amplified fragment length polymorphism (AFLP)**

AFLP was developed in the mid-1990s [49]. Briefly, it involves digestion of genomic DNA with two restriction enzymes (usually a frequent cutter and a rare cutter) followed by ligation of oligonucleotide adaptors to the sticky ends of the restriction fragments [50]. Adaptors with site restriction sequences serve as target for primer annealing, and the ligated products are then amplified under high stringency conditions. The procedure allows the selection of a subset of the restriction fragments. Typically, 50–100 amplified fragments are generated. To be visualized, these fragments need to be separated in high resolution electrophoresis systems (denaturing polyacrylamide gels). Fluorescent dye-labelled primers can be used allowing the detection of amplified fragments in gel-based or capillary DNA sequencers. This variant technique is referred to as fluorescent amplified fragment length polymorphism (FAFLP) allowing the highest resolution of all fragments of different size [51], [52]. AFLP usually involves two PCR steps. The first one consists of the preselective amplification using unlabelled primers with a single selective nucleotide in the primer. The reaction product is then diluted to obtain the adequate template concentration for the second PCR amplification in which additional selective nucleotides are added in the primer to improve specificity. During this latter amplification, the labelled primers are used. AFLP is a highly resolutive typing technique. Like RAPD, AFLP is a universal and multilocus marker technique that can be applied to genomes of any source without requiring any prior sequence information. However, AFLP is more reproducible than RAPD as it uses specific primers, and amplification is achieved under high stringency conditions [49], [50]. Although AFLP has proved to be reliable and reproducible as a genotyping method, it has been rarely used for C. albicans typing mainly because it is multiple-step, fairly expensive and requires a relatively high level of expertise [50],[52],[53].

**C) Exact DNA-Based Typing Methods .1. PCR-based Methods**

The invention of PCR was a landmark in the progress of molecular microbiology and has had a substantial impact on the diagnosis of infectious diseases. The key strongpoint of these techniques consists in the amplification and detection of minute amounts of microbial nucleic acid in the background of host DNA.

**1.1 Target and primer selection**

Generally, two strategies of PCR target selection can be adopted. If species-specific sequences are selected as primer-annealing sites, PCR will enable highly specific detection of just one pathogenic yeast species. On the other hand, when universal pan fungal sequences are targeted, PCR will result in amplicons in case any fungal DNA is present in samples. *Candida*-genus specific sequences can also be targeted to detect all *Candida* yeast species. If a broader spectrum of species is targeted, post-PCR analysis is necessary for subsequent species identification. To ensure high sensitivity of PCR detection, primers should preferentially target multicopy genes. Also,high specificity should be secured by targeting sequences specifically found only in the pathogen of interest. The ribosomal RNA (rRNA) gene appears to meet both of these criteria. A tandem array of 50 to 100 copies of the rRNA gene can be found in the haploid genome

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of all fungi. This consists of the small subunit rRNA gene (18S), the 5.8S gene and the large subunit rRNA (25S) gene,separated by the internal transcribed spacer regions, ITS1 and ITS2. While rRNA genes are highly conserved in fungi, ITS regions involve both highly variable and highly conserved areas [54], thus allowing the generation of species,genus or fungus specific primer.

**1.2 Nested PCR**

Nested PCR can be used to increase both the sensitivity and specificity of PCR detection. In this approach, two rounds of PCR are performed. In the first round, outer primers target a larger region for amplification. Amplicons from this round are then added as template into the second round reaction mixture, where inner primers target a fragment of the first round amplicon. Specificity of the assay is increased, because four primers have to anneal in an arranged fashion instead of just two in a single PCR.Sensitivity is increased, because addition of fresh reagents and dilution of first round amplicons in the second round mixture enables additional amplification of a fragment of the amplicons from the first round mixture. Two-rounds setting of nested PCR can also be used to combine the advantages of broad-range and species-specific targeting of yeast sequences. The outer primers can target universal sequences resulting in amplicons in a broad range of yeast species, followed by several parallel second round reactions with species-specific inner primer pairs. When second round primers are carefully designed to prevent interference, primer mixes can be used in a common reaction mixture to reduce costs, in an approach called multiplex PCR. Nested PCR approach was adapted for use in *Candida* species detection [55],[29] On the other hand, the extreme sensitivity of nested PCR results in its major drawback – the occurrence of false-positive results mainly due to the cross-contamination with previously amplified products and also due to contamination with environmental microorganisms, or even contaminated commercially available reagents. To avoid this pitfall, laboratories must follow stringent precautions such as establishing separate rooms and equipment for each step of the PCR and other procedures [56], [57].

**1.3. Real-time PCR**

The real-time polymerase chain reaction uses fluorescent reporter molecules to visualize the production of amplicons during each cycle of the PCR reaction. This is in contrast to endpoint detection in conventional PCR, where the amplicon is detected after completed amplification only. Real-time monitoring of amplification based on increase of fluorescence of reporter molecules enables quantification of the target DNA, because the time point at which the amplicons reach a specific fluorescence level during cycling corresponds with the starting amount of target DNA. This correlation is impossible in the case of conventional PCR, where the final amount of amplicons always reaches a uniform level due to inhibition of further amplification in the plateau phase of the reaction. The process of amplification can be monitored either using labelled probes which specifically hybridise to the newly formed amplicon molecules, or by staining newly formed double-stranded DNA molecules with non-specific dsDNA binding dyes (e.g. SYBR Green I, BEBO, LC Green or BOXTO). The use of probes increases the specificity of PCR, because an additional sequence homology between the amplicon and probe is necessary for successful reporting of amplification. When a dsDNA binding dye is used instead of a specific probe, melting analysis of the amplicon has to be performed subsequently to verify the identity of the amplicon. Sometimes, unambiguous differentiation between specific and non-specific products can be problematic. On the other hand, melting analysis can provide additional useful information about the amplified sequence. The use of an integrated thermocycler/fluorimeter with highly efficient heat exchange mechanism has significantly shortened the turnaround time of real-time PCR.Both amplification and detection take place in the same closed vessel, reducing post-amplification manipulation steps and dramatically decreasing the risk of false-positive results. Despite greater start-up expense and the lack of standardization, the oncoming explosion of new chemistries and instrumentation, sensitivity, reproducibility and potential for high-throughput, will nevertheless make the real-time PCR attractive and indispensable for future diagnostic mycology.Several studies have reported the identification of *Candida* species by targeting the rRNA gene complex using real-time PCR.[58],[26],[59]

**1.4. Post-PCR analysis**

Whether conventional or real-time PCR is used, several options for post-PCR analysis are available to characterize the amplicon and conclusions can be drawn on its species specificity, especially if universal sequences are targeted for amplification. Obviously, the only ultimate and most accurate way of post-PCR analysis is direct sequencing [60].However, alternative sequencing techniques, e.g. pyrosequencing, are under continuous development and promise

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further reduction of costs in the future. All the other techniques of amplicon post-PCR analysis rely in some way on characterization of its sequence-related variability. The length of the amplicon can be roughly estimated by agarose gel electrophoresis, which represents the most simple and traditional technique of post-PCR analysis, also called Amplified product length polymorphism (APLP). More accurate length characterization of amplicons can be achieved by polyacrylamide gel electrophoresis,which can be automated in a capillary-based analyser [61],[62].Restriction analysis of amplicons represents a rather cheap and elegant but laborious and more time-consuming technique. Similarly,single-strand conformational polymorphism (SSCP) can be employed to evaluate sequence-based characteristics of amplicons [63] but it is not widely used because of special expertise and labour needed for correct performance.To avoid the time-consuming and laborious electrophoresis step, used traditionally in the above-mentioned techniques, two alternative approaches can be applied. Microtitration plate enzyme immunoassay (PCR-EIA) can be utilized as a user-friendly alternative, which also improves detection sensitivity.

**2. Nucleic Acid Sequence Based Amplification (NASBA)**

NASBA is a specific and very sensitive RNA amplification technique, which exploits the action of three enzymes, i.e. reverse transcriptase, RNase H and T7 RNA polymerase, in an isothermal amplification process with cDNA as an intermediate [64],[65]. In medically important fungi,conserved regions of the 18S rRNA gene can be targeted by the amplification. Labelled oligonucleotide probes are then hybridised to an internal specific sequence of the *Candida* yeast species. Amplification and detection can be completed within few hours and the analysis has shown a detection limit of 1 CFU. NASBA has been evaluated to detect six various *Candida* species [66]. The main benefits of NASBA compared to PCR are no need of thermal cycling instrument and specific detection of living yeast cells, because RNA unlike DNA is rapidly degraded outside cells. The main disadvantage, which prevents more widespread use of NASBA, is the high price of the three enzymes mixture.

**3. Identification by Fluorescence in situ hybridisation (FISH)**

Fluorescent *in situ* hybridisation (FISH) with fluorescein-labelled oligonucleotide probes is a convenient way to detect yeasts without the need of pure culture. The employment of novel PNA (peptide nucleic acid) probes combines their high-affinity with advantages of targeting highly structured rRNA region, which has extended the potential of this method. Briefly, probes are hybridised to smears made directly from the contents of blood culture bottles on a slide, non-hybridised probes are washed out and slides are examined by fluorescence microscopy to reveal the presence of the organism. The sensitivity of the method has been estimated at least as similar to most results obtained by PCR-based assays [67] Due to a simple technical protocol with the exclusion of DNA extraction, the entire PNA FISH requires only 2.5 hours after a blood culture is designated positive by an automated blood culture system, the whole procedure is suitable for automation. FISH including probes specific for *Candida* species has been demonstrated to be a reasonable diagnostic tool for species identification [68]. PNA FISH has been developed to differentiate *C. albicans* from non-*albicans Candida* species [69] evaluated in a multicenter study and its implementation in hospital reduced antifungal drug expenses The same group also conducted a PNA FISH assay to differentiate *C. albicans* from *C. Dubliniensis [70]*

Most of the techniques described above have proven to be effective and reliable typing methods for the investigation of the C. albicans epidemiology at local level. These techniques are suitable for clinical studies in individual laboratory settings, but the exchange and comparison of data between laboratories are difficult, if not impossible. At present, only two techniques lend themselves well to this exchange because they generate unambiguous results with an excellent reproducibility. For this reason, for genotyping *Candida albicans* isolates, two PCR-based methods have recently emerged: called exact DNA-based typing methods. They include microsatellite length polymorphism (MLP) and multilocus sequence typing (MLST)

**4. Microsatellite length polymorphism (MLP)**

MLP typing is a PCR-based system that exploits the high variability in the repeat number of microsatellite sequences, defined as tandemly repetitive stretches of two to six nucleotides. Microsatellite markers consist of a defined primer pair flanking a specific microsatellite region in the genome. The PCR fragments amplified differ in length according to the number of repetitions of the microsatellite stretch. Microsatellites display a high polymorphism level and a mendelian codominant inheritance and thus can serve as excellent candidates for genetic analysis. For each isolate,

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MLP typing identifies the presence of one (homozygous) or two (heterozygous) different fragments, or alleles, at a given locus. As microsatellites are highly mutable sequences, one concern with MLP approach is that alleles may be identical not by inheritance but by mutation, resulting in homoplasy [71], [72]. Fluorescently labelled primers are used to amplify specific loci, and the length of the alleles is measured by migration of the PCR products in a high-resolution gel electrophoresis achieved by an automatic sequencer [73]. The lengths of the alleles are numeric data and can be easily compared.

MLP is easy to perform, rapid and is amenable for automation and high throughput. Overall, MLP is one of the most discriminative methods for C. albicans typing. However, its resolving power depends on the microsatellite marker used. Several polymorphic microsatellite loci (e.g. EF3, CDC3, HIS3, ERK1,2NF1, CCN2, CPH2, EFG1, CAI and CAIII to CAVII) have been identified in the C. albicans genome, exhibiting an unequal discriminatory power ranging between 057 (for CAV) and 097 (for CAI) [73],[74]. In contrast to conventional techniques where the number of specimens tested in the same run is limited by the constraints of conventional electrophoresis, MLP analysis proved to be very suitable for application in large-scale epidemiological studies as up to 96 samples can be analysed in a single run. [74].MLP is a robust technique and strongly recommended for epidemiological studies of C. albicans and other fungi such as fumigatus and C. neoformans [75],[76]. However, inherent drawbacks of MLP include the high cost of specialized equipment and the difficulty of implementing the technique in routine use, especially in developing countries.To overcome this difficulty, Li and Bai described single strand conformation polymorphisms (SSCP) of the microsatellite CAI and showed that SSCP analysis of this microsatellite marker is a powerful (discriminatory power of 0993) and cost-effective approach for rapid strain typing of C. albicans in clinical laboratories, especially in the detection of microevolutionary changes . This technique was later used for accurate typing of vulvovaginitis C. albicans isolates [77]

**5. Multilocus sequence typing (MLST)**

MLST is typically based on the analysis of nucleotide sequence polymorphisms within the sequences of internal fragments of six to eight independent genes (loci). Genes chosen for MLST analysis are generally those with housekeeping functions that are subject to stabilizing selection.In addition, selected loci must provide as much sequence diversity as possible to allow high levels of allelic discrimination [15]. MLST involves amplification of DNA fragments (400–500 bp) by PCR followed by DNA sequencing. For each housekeeping locus, different sequences are considered as distinct alleles. Each isolate is therefore characterized by a series of alleles at the different loci that correspond to the multilocus sequence type. Data generated by this DNA sequence analysis are unambiguous and can be stored and readily accessible in databases [15], [78]. In contrast to MLP, MLST proved to be highly reproducible between laboratories and thereby liable to standardization and portability [78]. It allows the exchange of genotyping data and the construction of international databases accessible via Internet. Online global databases for many microorganisms are currently available at www.mlst.net, including data from epidemiological studies carried out worldwide. This permits global epidemiological and population analysis. MLST methodology was first developed and used for typing pathogenic bacteria and later some pathogenic fungi (e.g. Cryptococcus gatti, Fusarium solani and Batrachochytrium dendrobatidis) [79], [80]. The technique was applied to C. albicans in the early 2000s. The first protocol,based on the analysis of six loci and a second one, based on the analysis of eight loci, were developed by Bougnoux et al. in 2002 [15] and Tavanti et al. in 2003 [81], respectively. Later, an optimized protocol based on a set of seven loci was proposed as an international consensus unifying the MLST scheme for C. albicans [82]. Results from strain typing using this system are shared through public Internet-linked database (http://calbicans.mlst.net/.) where data have been accumulated from different geographical locations. This database provides an interesting source to evaluate the worldwide diversity of C. albicans and the relationships of isolates identified at various locations. It is worth mentioning that MLST is the only typing method that has a public database, not only for C. albicans but also for C. tropicalis, C. glabrata, C. dubliniensis and C. krusei. In contrast, C. parapsilosis shows too little sequence diversity to be typeable by MLST [78]. Because C. albicans is diploid, nucleotide sequences generated by the MLST analysis are likely to show heterozygosity at polymorphic sites, and therefore,strains are unambiguously characterized by a diploid sequence type (DST) .MLST analysis has been successfully applied to population genetics and molecular phylogeny studies of C. albicans [6],[15],[78]. In population genetic studies, MLST data have confirmed previous studies in the field and, in some instances, refined our understanding of the epidemiology of C. albicans [6].Population genetic

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analysis of C. albicans MLST data concerning isolates obtained from separate sources showed that the species could be divided into a large number of clades and that clades differed in the proportions of isolates they included according to the geographical origin and the anatomical sources.

Candida albicans population structure as determined by MLST typing showed a good correlation in the clustering obtained with Ca3 fingerprinting, with clades I, II, III and SA delimited by Ca3 probe. However, clade E as identified by Ca3 fingerprinting has been separated by MLST analysis into various clades (. Limitations of MLST in the characterization of unrelated strains may be explained by the fact that (i) the method analyses the sequences of only seven 300–400bp loci, so that isolates with identical DSTs may differ substantially through large genomic rearrangements in regions that do not encompass the sequenced loci and (ii) the diploid nature of C. albicans can result in two strains yielding identical DSTs, even though they may differ in the organization of the heterozygous bases at the polymorphic sites [78],[82]. An additional problem raised by the C. albicans MLST analysis is that three of the chromosomes of C. albicans (3, 5 and 7) are not represented in the consensus scheme. Lott and Scarborough developed SNP analysis by microarray to complement MLST typing and to extend the SNPs to all the yeast chromosomes. The array consists of multiple replicates of 79 SNPs, derived from 19 loci located on all eight chromosomes, including the seven genes (57 SNPs) that comprise the MLST consensus scheme. The remaining 22 SNPs are from 12 additional loci located on the remaining chromosomes [9]. Further studies are needed to assess the input of the system in the epidemiology and population genetic studies of C. Albicans. Both MLP and MLST fulfill several criteria for a broadly useful tool for genotyping *C. albicans* isolates. First, all the isolates were typeable by both methods. Second, the variability within the selected sequence was sufficient to differentiate 50 unrelated isolates and the discriminatory power was >0.99 for both methods. Third, the resulting data can be stored in digital form for subsequent analysis with the aid of specialized software. A theoretical disadvantage of both methods is that only very small regions of the *C. albicans* genome are analyzed, in contrast to techniques such as RAPD, which potentially examines the entire genome.

**6. DNA-microarrays**

Microarray-based systems offer an attractive outlook not only for the future of strain typing. They a offer high level of sensitivity, specificity and throughput capacity, without requiring *a priori* knowledge of specific sequences.Chips or microarrays are high-density microscopic sets of oligonucleotide probes immobilized on solid surface,to which nucleic acid samples are hybridised. Perfectly matched sequences from the sample hybridise more efficiently to the corresponding oligomers on the array and give stronger signal than mismatched bound sequences.The final signal is detected by high-resolution fluorescent scanning and analysed by computer software,thus enabling automation and standardization.[83] Easier management of the vast data generated and reduction of the costs of DNA-chips are only a matter of time. Then,microarrays surely will move from the research area to clinical practice. For typing purposes, microarrays can be directed to identify the presence and quantity of different sequence variants of specific genes or regions, e.g.rRNA genes, internal transcribed spacers (ITSs) in particular. Ongoing sequencing projects in pathogenic yeasts will also soon enable quite straightforward designing of whole-genome DNA microarrays [84].The use of microarrays for microbial fingerprinting has been already reported for *Salmonella enterica* isolates, for closely related *Xanthomonas* pathovars and *Mycobacterium* species[85],[86].Rapid automated performance of tens of thousands of hybridisation assays on a tiny chip represents the strongest point of this technology.

## IV. CONCLUSION

Both conventional DNA-based methods and exact DNA-based methods have provided useful insights into the epidemiology and population structure of C. albicans and non albicans with other yeast species.The major drawback of the conventional methods lies in their lack of standardization, reducing their potential for interlaboratory comparisons and therefore global population studies; but these methods are very suitable to investigate epidemiological trends at a local level. Exact DNA-based methods, including PCR based methods , MLP and MLST, have emerged as very efficient typing tools. The main advantage of these methods is that generated data are unambiguous and highly reproducible and can be stored in databases offering an unprecedented degree of portability and accessibility to all interested users. Such techniques are much more appropriate for global epidemiology. At present, MLST is the only typing method that has a public database and represents the most powerful approach for phylogenetics of C. albicans,

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whereas MLP analysis needs further standardization.

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