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Review Article

Identification of Candida Species in the Clinical Laboratory: A Review of Conventional, Commercial and Molecular Techniques

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ABSTRACT

The incidence of Candida infections has increased substantially in few decades due to the increased use of broad-spectrum antibiotics, immuno-suppressants and indwelling devices. The intensive care unit hospitalisation and other immunocompromised states has further attributed to high rates of invasive Candidiasis. Among Candida species, *C. albicans* accounts for the majority of superficial and systemic infections. Both *C. albicans* and Non albicans Candida is associated with high morbidity and mortality. Many preliminary methods are used for isolation and identification of Candida species. The isolation and identification of Candida species in Mycological laboratory with conventional methods is time consuming and cumbersome as it requires 3 to 6 days. Conventional methods are technically hard to perform and do not give genotypic level of identification. Therefore use of rapid, automated, advanced and molecular methods would help researchers to develop new therapeutic interventions and assist in better patient management. Now a days, a wide range of molecular techniques have been used including non-DNA-based methods and DNA-based methods because they generate unambiguous and highly reproducible typing data. In this review, all the diagnostic methods used for Candida are summarized, and their advantages and limitations are discussed with regard to their application to patient care.

Key Words: *Candida albicans*, Non albicans Candida, Phenotypic methods, Molecular methods.

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INTRODUCTION

The incidence of Candidiasis has increased in recent years due to the widespread use of broad-spectrum antibiotics, the growing numbers of HIV-infected and other immunocompromised states.[1] Despite the predominance of *Candida albicans*, non-albicans Candida species such as *Candida glabrata, Candida tropicalis, Candida guilliermondii, Candida dubliniensis, Candida parapsilosis, and Candida krusei* are emerging as colonizers and cause both superficial and systemic infections.[2] Some of these species are intrinsically resistant to antifungal agents. *C. glabrata* and *C. krusei* are innately resistant to the commonly used antifungal agent like azoles.[3] Consequently, rapid and correct identification of Candida species can play an important role in infection management and decreasing mortality rates. Additionally, there are many reports of atypical isolates of *C. albicans* in the last few years.[4] Wide range of Phenotypic, Chromogenic, Automated, Rapid and Molecular methods are adopted for the identification of Candida. The earliest methods used in the typing of *C. albicans* and non albicans were based on phenotypic characteristics including morphotyping, biotyping, serotyping, resistance to various chemicals etc. However, phenotypic techniques have a very low degree of discrimination and reproducibility, which obviously constitutes a limitation for reliable diagnostic and epidemiological analysis. Molecular identification methods are becoming popular due to their accurate results, high sensitivity and specificity. The advent of the molecular DNA-based techniques revolutionized the knowledge on the biology and epidemiology of Candida.[5-6]

AIMS

- To describe the diagnostic techniques both phenotypic as well as genotypic used for identification of Candida species.
- To evaluate their strengths and limitations.

METHODS FOR THE IDENTIFICATION OF CANDIDA SPECIES

I) PHENOTYPIC METHODS

(A) Conventional methods

1. Germ-tube test

The germ-tube test is considered a simple, economical, and efficient procedure for differentiating *C. albicans* from other Candida species. It is based on the observation that *C. albicans* produces tube-like structures (called germ tubes) when incubated in serum within 2–4 h at 37°C. A germ tube or pseudohyphae is a non-septate extension of the yeast cell that differs from true hyphae by having no constrictions at the point of connection to the cell. Germ-tube test provides a rapid identification test for *C. albicans*.[7]

2. Chlamydospore formation

Chlamydospores formation is a peculiarity of *C. albicans*. In fact, with the Dalmau techniques, large, highly refractile-walled chlamydospores may be seen as terminal or on short lateral branches in *C. albicans*.[8]

They are thought to be dormant growth forms that arise under conditions of nutrient depletion. Although cornmeal and rice extract agar are the best-known chlyamdospore-inducing media[9], a number of other growth media have been suggested and indicated for chlyamdospore formation such as casein agar. It is a useful test to discriminate between *C. dubliniensis* and *C. albicans*.[10]

3. Carbon and Nitrogen assimilation

Substrate assimilation is based on the growth of an organism in the presence of chemically pure substrates, and it is widely used for the identification of yeasts. The ability of a particular isolate to assimilate a specific carbohydrate as the sole carbon source has been conventionally used in Candida identification. The popularity of this test can be attributed to the relative simplicity of the method, the low cost, and the ready availability of standardized protocols.[11] Assimilation properties can be determined by two different methods, both utilizing a basal medium (a yeast nitrogen base) that support the growth of yeasts when an appropriate substrate is added. In the first auxanographic method, sugar solutions are allowed to diffuse from wells cut in a gel that contains the necessary nutrients except carbon. A circular zone of growth around the wells indicates assimilation of the carbohydrate substrates however, it is considered time consuming. In the second method, growth of Candida is observed in tubes containing a synthetic basal medium with different carbon sources. The tubes are incubated and examined for growth. Nitrate assimilation can be tested in broth medium using alpha naphthylamine and sulfanilic acid reagents. This method is preferred due to its high sensitivity and specificity.[12]

4. Carbohydrate fermentation

Fermentation tests are generally performed in liquid media and are based on demonstration of acid and/or carbon dioxide production. Although carbohydrate fermentation is a useful test for differentiating Candida species, this test is considered less sensitive and hence less reliable than the carbohydrate assimilation test.[13] Individually, these physiological tests are time consuming and laborious to perform, particularly in routine diagnostic laboratories.

(B) Rapid Identification systems

Several commercially available simplified diagnostic systems have been developed based on germ-tube test, chlamydospores formation, and sugar assimilation tests. These commercial methods often represent a miniaturized version of conventional tests, allowing more convenient and rapid identification of Candida species. The most common commercial methods are discussed below:

1. API 20C Aux system

The API 20C system was one of the first commercial identification kit introduced for rapid yeast identification. This system is no longer available and has been replaced by the API 20C Aux (bioMerieux Vitek, Hazelwood, MO, USA). This system is comprised of 19 carbohydrate assimilation tests in a cupule format. After incubation at 30°C (24, 48, and 72 h), the turbidity of cupules can be assessed and a profile number is generated. Final identification is accepted when the computer-assisted numerical approach categorizes the profile number as excellent, very good, good, or acceptable. In general, the API 20C Aux seems to be accurate in differentiating Candida species, with identification rates higher than 85%.[14] It is suggested that C. dubliniensis could be differentiated from C. albicans on this medium due to its inability to assimilate Xylose and alpha- methyl-D-glucoside.[15] On the other hand, studies have demonstrated problems with the ability of the API 20C Aux system to correctly identify *C. krusei*. Precision is required during standardization and inoculum seeding, correct interpretation of the turbidity level requires skill, and readout times can be up to 72 h.[16]

2. API Candida system

The API Candida system (bioMerieux, Marcy l'Etoile, France) is based on the detection of enzyme reactions and acidification of sugars. The system consists of a strip of 10 tubes, which permits 12 identification tests: five carbohydrate acidification tests and seven enzymatic tests.[17,18] It is designed for the identification of 15 yeast species after 18–24 h of incubation at 35°C .The reactions are visually interpreted by spontaneous color changes without addition of reagents, as in many API systems. Occasional errors in identification of yeasts using the API Candida system could be avoided if morphological characteristics are taken into account within the numerical profile.[18]

(C) Chromogenic media-based commercial systems

1. CHROMagar

CHROMagar is a selective and differential medium for isolation and identification of Candida species. CHROMagar medium contain chromogenic substrates which react with enzymes secreted by specific Candida species to yield colonies of varying colours. CHROMagar is an attempt for rapid and simple identification of Candida species.[19]

2. Fluorogenic membrane filtration method

This method is an improved chromogenic method deals with sensitivity and speed of enzymatic systems for detection of yeasts by an unusual two-step method consisting of micro-colony formation on a nylon membrane filter followed by an assay of enzyme activities using fluorogenic substrates in the presence of a membrane permeabilizer. This method allows detection of relevant members of Candida species, such as *C. albicans,*

C. glabrata, C. krusei, and C. tropicalis, within 9–11 h. This modified CHROMagar procedure increased the ability to detect Candida species.[20]

3. Candida ID system

The Candida ID System (bioMerieux, Marcy l'Etoile, France) is based on a chromogenic indolyl glucosaminide substrate, which is hydrolyzed by *C. albicans* to give a turquoise or blue insoluble product. The colonies of *C. tropicalis, C. lusitaniae,* and *C. guilliermondii* appear pink on this medium, and the other Candida species are white. On this medium, identification of *C. albicans* seems to be more rapid than with CHROMagar.[21]

4. Baxter Micro-Scan yeast identification panel

The Baxter Micro-Scan YIP (Baxter MicroScan, W. Sacramento,Calif., USA) is a 96-well microdilution plate with 27 dehydrated substrates comprised of 13 amino acid, ßnaphthylamides, nine nitrophenyl-linked carbohydrates, three carbohydrates, urea, and indoxyl phosphate. The system has the advantages of providing identifications within 4 h, plates are easy to inoculate, and positive reactions can be readily determined without prior experience.[22]

5. Uni-Yeast-Tek system

The Uni-Yeast-Tek kit (Flow Laboratories, Inc., Baltimore, Md., USA) consists of a multi-sectioned dish containing carbon assimilation agars, urea agar, nitrate assimilation agar, a central well containing Corn-meal with Tween 80, a broth containing 0.05% glucose and 2.6% beef extract, and a wheeled classification key for the identification of 16 common clinical yeast isolates. For test purposes, each well is inoculated with one drop of a distilled water suspension of the yeast. The carbohydrate reactions are observed for a purple to yellow colour change (positive reaction), and the nitrate medium for a yellow to blue–green colour change. At present, the major advantage of using the Uni-Yeast-Tek system in clinical laboratories is its rapidity, since a single technician can inoculate 50–75 plates in a single session. However, a major drawback is that the system requires monitoring of yeast growth for up to 6 days before a definitive identification can be established.[23]

6. Fungichrom I and Fungifast I twin systems

The Fungichrom I and Fungifast I twin (International Microbio, Parcd'activites-allee D'athenes, France) systems consist of 16 wells and 10 test cupules, respectively. In both systems, color changes are examined after incubation for 24–48 h at 30°C. Fungichrom I is an appropriate system for non-specialized clinical microbiology laboratories due to its simplicity and relatively good identification rate (85%). Strains of *C. albicans, C. glabrata*, and *C. parapsilosis* were identified by this system without any additional tests. They also reported that Fungifast system is not so objective and/or rapid as the Fungichrom I system.[24]

7. BiGGY agar system

BiGGY agar (Oxoid Company, Wade Road, Basingstoke, Hampshire, UK) is a chromogenic medium that leads to production of brown to black colonies by the extracellular reduction in bismuth sulfite to bismuth sulfide. *C. albicans* and *C. tropicalis* strains appear as light brown and dark brown colours, respectively, that are hard to differentiate when grown on BiGGY agar. *C. krusei* produces typical large, rough, dark brown colonies with surrounding yellow zone, and *C. parapsilosis* grows as light brown-greenish, grey- cream colored colonies in BiGGY agar.[25]

(D) Automated Identification system

1. Vitek YBC system

The Vitek system (bioMerieux Vitek, Inc., Hazelwood, MO, USA) The system was subsequently modified and introduced to the clinical laboratory as the Auto-Microbic System. It consists of a 30-well, disposable plastic card containing 26 conventional biochemical tests and four negative controls, for identification of 27 species of medically important yeasts within 22–24 h. The Yeast Biochemical Card (YBC) is one of several testing packages used with the Vitek System, which includes a programmed computer, a reader–incubator unit, a filling module, a sealer module, and a printer.

Generally, common clinical isolates such as *C. albicans, C. tropicalis, C. parapsilosis,* and *C. glabrata* have been identified with a high level of confidence. The advantages of the system include minimal set-up time, automated inoculation of substrates with the same suspension of each isolate, elimination of test variability, ability to perform 26 biochemical tests simultaneously from the same inoculums, and computer-assisted identification and easy updating of computer software to reflect changes in the biochemical profiles of isolates and to increase the number of organisms identified.

Disadvantages include no manual backup because reaction thresholds are not visually discernible, the need for a large inocula, limitation of the database, storage space for the disposable injector tubes,and the short shelf life of YBC.[26]

2. The Vitek 2 ID-YST system

It is a second-generation automated system comprised of 64 wells with 47 fluorescent biochemical tests. The latter is made up of 20 carbohydrate assimilation tests, six organic acid assimilation tests, eight substrates for detecting the oxidases coupled with 4-methylumbelliferone, nine substrates specifically for arylamidases coupled with seven amino-methylcoumarin, and four miscellaneous tests. The Vitek 2 system is mainly useful for identification of *C. glabrata* and *C. dubliniensis.* Identification of the yeasts within 15 h due to the very sensitive fluorescence technology represents a major advantage of the new Vitek 2 system. However, one drawback is that use of cultures older than 24 h leads to suspicious results.[27]

II) SEROLOGICAL METHODS

Other commercial tests such as serological tests have been evaluated for their performance in the diagnosis of invasive Candidiasis.

1. (1, 3)-beta-d-glucan

A new fungal surrogate marker, (1, 3)-beta-d-glucan, can be used for surveillance and diagnosis of invasive fungal infections. (1, 3)-beta-d-glucan testing aids the diagnosis of an invasive fungal infection as well as monitor the response to treatment. This test has showed both sensitivity and specificity. It was observed that (1, 3)-beta-d-glucan test was superior for the diagnosis of candidemia relative to other tests evaluated.[28]

2. Galactomannan

Galactomannan, a polysaccharide fungal cell-wall component, can be detected in the blood during invasive fungal infections and can also be used for prognosis and to monitor response to therapy. In addition, to optimize detection, the assay is usually performed twice per week during the interval of greatest risk.[29]

Table 1: Methods used for the identification of Candida species

I) PHENOTYPIC METHODS	III) MOLECULAR METHODS
A. Conventional methods	**A. Non DNA based methods**
1.Germ-tube test	1. Multi-locus enzyme electrophoresis
2.Chlamydospores formation	**B. DNA Based methods**
3.Carbon and Nitrogen Assimilation	• Conventional DNA based methods
4. Carbon Fermentation	• Exact DNA based methods
B. Rapid Identification Systems	**a. Conventional DNA based methods**
1. API 20C Aux system	1. Pulsed-field gel electrophoresis
2. API Candida system	2. Restriction enzyme analysis (REA)
C. Chromogenic media-based systems	3.Random amplified polymorphic DNA (RAPD)
1. CHROMagar	4. Amplified fragment length polymorphism (AFLP)
2.Fluorogenic membrane filtration method	**b. Exact DNA based methods**
3.Candida ID system	1. PCR based Methods
4.Uni-Yeast-Tek system	• Nested PCR
5. Baxter MicroScan yeast identification panel	• Real Time PCR
6. Fungichrom I and Fungifast I twin systems	2. Nucleic Acid Sequence Based Amplification (NASBA)
7. BiGGY agar system	3. Fluorescent in situ hybridisation (FISH)
D. Automated methods	4. Microsatellite length polymorphism(MLP) typing
1. Vitek YBC system	5. Multilocus sequence typing (MLST)
2. Vitek 2 ID-YST system	6. DNA-microarrays
II) SEROLOGICAL METHODS	**IV) NEWER METHODS**
1. (1,3)-beta-d-glucan	Matrix-Assisted Laser Desorption Ionization –Time Of
2. Galactomannan	Flight mass spectrometry (MALDI-TOF MS)

III) MOLECULAR METHODS

Molecular identification methods are becoming popular due to their high accuracy, sensitivity and specificity for the identification and differentiation of *C. albicans* from other Candida species. For molecular identification, several procedures have been proposed to detect and differentiate Candida species both by non-DNA-based methods and DNA based methods, which include conventional DNA-based methods and exact- DNA-based methods.

A) NON DNA BASED TECHNIQUES

1. Multi-Locus Enzyme Electrophoresis

MLEE 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 may provide valid measures of genetic distance because it assesses defined multi locus differences and information relevant to population genetic studies as well as to epidemiology. MLEE is a reliable technique with a relatively high discriminatory power in the distinction between unrelated strains and shows good reproducibility. It outperforms many of the DNA-based methods and remains a useful tool for molecular investigation of natural populations of C. albicans. The major drawback of the MLEE is that it assays the genome indirectly and evaluates variations accumulated very slowly in the species. Also, 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.[30,31]

B) DNA-BASED METHODS

It includes

a. Conventional DNA-Based Methods and

b. Exact DNA-Based Typing Methods

A. Conventional DNA-Based Typing Methods

1) Pulsed-field gel electrophoresis (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), contour-clamped 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 chromosome-sized DNA molecules, the analysis of the chromosomal banding patterns, known as electrophoretic karyotypes, and the detection of karyotypic variations within the species. Briefly, cells are mixed

with enzymes to remove the cell wall and then embedded in an agarose plug, which protects the large DNA molecules from shearing forces. Protease and detergent are added to remove membranes and digest proteins. The yeast chromosome-sized DNA fragments are separated according to size and then visualized by ethidium bromide staining. Assessment of differences in banding patterns can be performed visually or using computer-assisted methods.[32,33]

2) Restriction enzyme analysis (REA)

REA was widely used in the earliest epidemiological investigations of C. albicans infection. 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.[34]

3) Random amplified polymorphic DNA

The random amplified polymorphic DNA (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. 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 relatively complex patterns that greatly vary among unrelated isolates. RAPD has been extensively used for typing of *C. albicans* and other Candida species.[35]

4) Amplified fragment length polymorphism (AFLP)

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 (Ballet al. 2004). 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. AFLP is more reproducible than RAPD as it uses specific primers, and amplification is achieved under high stringency conditions .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.[36,37]

B. 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 fungal diseases. The strong point of these techniques consists in the amplification and detection of minute amounts of microbial nucleic acid in the background of host DNA.

Target and primer selection

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 fungal 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 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 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, thus allowing the generation of species, genus or fungus specific primer.[38]

1) Nested PCR

This technique consists of a two sets of primers for specific amplification of the DNAs of Candida spp. that are used in two successive runs of PCR to improve the specificity and sensitivity of Candida identification. First, outer primers target a larger region for amplification. Then, the real amplification of the target sequence is followed using the product from the former reaction. Nested PCR is considered a very specific technique because if the wrong PCR fragment was amplified in the first run, there is a very low probability that the region would be amplified again by the second pair of primers. It can be noted that if the second round of primers are carefully designed to prevent interference, primers mixes can be used in a common reaction mixture to reduce the costs of using the multiplex PCR methodologies. Results from using a rabbit model of experimental candidemia demonstrated that the sensitivity of nested PCR for diagnosis of systemic candidiasis was higher in serum samples than in whole blood.[39]

2) Real-time PCR

The real-time PCR (RT-PCR) is a variant of the standard PCR technique that allows real-time quantification of the amplified DNA, at each cycle of the PCR. There are several fluorescence methods that can be used in real-time PCR, which can be grouped in two major classes, according to the type of the fluorescent compound and its behaviour during the process. Thus,

the process of amplification can be monitored either using labelled probes, which specifically hybridize to the newly formed amplicon molecules, or by staining newly formed double-stranded dyes. The use of probes increases the specificity of PCR because an additional sequence homology between the amplicon and the probe is necessary for successful reporting of amplication. In fact, recent studies have suggested real-time monitoring of the RT-PCR using different technologies to measure the gene expression more rapidly and accurately.[40]

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. However, alternative sequencing techniques, e.g. pyro-sequencing, is under continuous development and promise 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. More accurate length characterization of amplicons can be achieved by polyacrylamide gel electrophoresis, which can be automated in a capillary-based analyser. 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 but it is not widely used because of special expertise and labour needed for correct performance.[41]

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. 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. 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.[42]

3. Fluorescent 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. 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. PNA FISH has been developed to differentiate C. albicans from non-albicans Candida species evaluated in a multicenter study and its implementation in hospital reduced antifungal drug expenses.[43]

4. MLP typing

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, MLP typing identifies the presence of one (homozygous) or two (heterozygous) different fragments, or alleles, at a given locus. 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. 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. MLP is a robust technique and strongly recommended for epidemiological studies of *C. albicans*.[44]

5. Multilocus sequence typing

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. 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 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 databases.[45]

6. DNA-microarrays

Microarray-based systems offer an attractive outlook of strain typing. It offers high level of sensitivity, specificity without requiring a prior 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. 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. Ongoing sequencing projects in pathogenic yeasts will also soon enable quite straightforward designing of whole-genome DNA microarrays.[46]

IV) NEWER METHODS

Matrix-Assisted Laser Desorption Ionization: Time Of Flight mass spectrometry (MALDI-TOF MS)

(MALDI-TOF MS) has emerged as a rapid, reliable, and cost-effective alternative for yeast identification becoming a powerful and gradually widely available tool in clinical microbiology laboratories. This method relies on the generation of microorganism 'protein fingerprints' that are compared with reference spectra in a well-characterized library. The operational procedures have been described. In summary, fungi are submitted to a thermal degradation by Curie-point pyrolysis. The resulting small molecules are then cleaved at their frailest points producing volatile fragments (pyrolysate). A mass spectrometer can separate the components of the pyrolysate on the basis of their mass-to-charge ratios to produce a pyrolysis mass spectrum. Therefore, this spectrum can be then analyzed as a chemical fingerprint of the yeast. This technique has been especially effective for interstrain comparison of a diversity of medically important microorganisms. It offer shorter turn-around times, and it also proved be more accurate comparatively to conventional methods. A MALDI-TOF score-oriented dendrogram is then created using default settings in Biotyper.[47]

CONCLUSION

A rapid and accurate identification of the disease-causing species of Candida is crucial for treatment. Premature diagnosis of invasive fungal infections is problematic because most clinical signs and symptoms are non-specific which delays the late initiation of effective antifungal therapy. Conventional methods have long been used as benchmark identification procedures for Candida species. However, these methods are laborious, time-consuming, and not reliable in identifying the broad spectrum of Candida species and usually require additional tests. Conventional methods have many limitations which lead to the development of several commercial systems which produce rapid yeast identification within 1.5–72 h. Although these systems have been extensively used for Candida identification, their use is limited, and some species cannot be identified and differentiated. Today, molecular strategies, such as PCR- or non-PCR based methods have been used as complements to conventional methods and providing more accurate results in less time (1.5–3 h). Given the high accuracy and speed with which molecular typing techniques can be carried out and rapid advances in technology, it is likely that most of these methods could improve routine clinical laboratory identification of Candida species. However, further studies are needed for standardization of such technical procedures.

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