Chapter

Laboratory Diagnosis of Candidiasis

Benson Musinguzi, Obondo J. Sande, Gerald Mboowa, Andrew Baguma, Herbert Itabangi and Beatrice Achan

Abstract

The burden of Candidiasis continues to increase and so does the *Candida* species. Although *Candida* species are closely similar phenotypically, they differ from each other in terms of epidemiology, genetic characteristics, antifungal susceptibility and virulence profile. Therefore, reliable and accurate laboratory methods for identification of *Candida* species can determine the Candidiasis burden and enable the administration of the most appropriate antifungal drug therapy to reduce fungal mortality rates. Conventional and biochemical methods are often used in identification of *Candida* species. However, these techniques are specific and sensitive enough in detecting the non albicans candida (NAC) species. Molecular techniques have improved the laboratory diagnosis and management of Candidiasis due to improved sensitivity and specificity threshold. This chapter provides an overview of different laboratory methods for diagnosis of Candidiasis.

Keywords: *Candida*, identification, candidiasis, laboratory, diagnosis, non-C. *albicans*

1. Introduction

There is a global raise in the burden of Candidiasis among immunocompromised individuals and this has to an increase in *Candida* species [1]. These species include both *C. albicans* and non *C. albicans* (NAC); *C. glabrata, C. tropicalis, Candida krusei, C. dubliniensis, Candida parapsilosis, Candida guilliermondii, Candida famata, C. kefyr, Candida norvegensis, Candida sake, Candida lusitaniae, C. pintolopesii, C. pseudotropicalis, C. globosa, C. dattila, C. inconspicua, Cobitis hellenica, Calamagrostis holmii, C. pulcherrima, C. valida, Candida fabianii, C. cacaoi, Candida zeylanoides* among [2, 3].

The phenotypic appearance of *Candida* species are relatively similar, however, different species differ from each other in terms of antifungal sensitivity, epidemiological distribution, genetic makeup and virulence attributes [4]. The diagnosis of Candidiasis is often clinical and empirical management is no longer adequate. This is partly due to misdiagnosis and varied antifungal susceptibility profile of the different *Candida* species [5]. This has worsened with the ever-increasing taxonomical shift in the etiology of Candidiasis towards resistant non albicans candida (NAC) [6]. This is partly caused by laboratory diagnosis which is frequently based on the conventional phenotypic and biochemical methods that are often not specific and sensitive in detecting NAC species [7]. However, diagnostic approaches have improved over the

years with the invention of advanced molecular techniques [8]. This chapter provides an overview of the laboratory methods for diagnosis of Candidiasis.

2. Laboratory diagnosis of candidiasis

The laboratory diagnosis of the Candidiasis involves the use of both Conventional (phenotypic) and molecular (genotypic) methods to detect visible and genetic characteristics of Candida respectively.

2.1 Conventional methods

Conventional methods are still commonly used for diagnosis and identification of fungi. These techniques are based on microscopic examination and fungal culture. Oral swab is collected, followed by microscopy and culture on selective media [4]. Microscopy can be done directly either from fresh samples or from fungal cultures. However, microscopy is non-specific, as different species can show the same morphological patterns and it is not possible to identify the *Candid*a species causing the Candidiasis [9]. Swab culture is normally the first test that is commonly done for identification of *Candida* species causing Candidiasis. However, It takes 1 to 3 days to have results [4]. Once positive cultures are available, other methods can be used to identify species of *Candida*. For instance, CHROM agar is a selective and differential medium for the identification of *Candida* species and can be used to identify *C. albicans, C. parapsilisis*, *C. dubliniensis*, *C. tropicalis* and *C. krusei*. It is widely used in mycology and it is found to be an effective primary identification test, where each species gives different colors of the colony forming units when species-specific enzymes split the chromogenic substrates [10]. Discrepancies may occur due to variations in the enzymatic reactions within the same *Candia* species [11]. *C. albicans* can be presumptively identified using the germ tube test; *C. albicans* shows a distinctive, tube-like structure when incubated in serum for 2–4 hours at 37°C. However, a possible limitation about the germ tube test is that some other *Candida* species such as *C. dubliniensis* also show a positive test result [12]. However, an easy and rapid commercialized latex agglutination test, Bichro-Dubli Fumouze® (Fumouze Diagnostics, France) has been evaluated to differentiate *C. albicans* from *C. dubliniensis* by detecting specific antigens located on the surface of *C. dubliniensis* blastoconidia [13]. In addition, automated biochemical and assimilation tests such as API and VITEK (BioMerieux Vitek, Inc., Hazelwood, USA) have been developed for *Candida* species identification. The API 20C system (Analytab Products, Plainview, USA) was one of the first available commercial kits used for the identification of yeast [12]. The ID 32C system (bioMérieux, France) has 12 substrates more than API which can enable identification of a diverse set of clinically important yeasts and can also differentiate between *C. albicans* and *C. dubliniensis* [14]. The Vitek 2 system is able to identify and detect *Candida* species and their antifungal susceptibility profile [15]. The main concern of these tests is that they require isolated fungal colonies and an incubation time of 2 to 3 days and misidentification of *Corynebacterium auris* may occur [16].

Indirect nonculture-based methods are available such as *C. albicans* germ tube antibody (CAGTA), circulating (1,3)-ß-D-glucan (BDG) antigen detection, mannan and anti-mannan antibody tests [17]. Much as, BDG Fungitell assay (Associates of Cape Cod, Inc) has been approved by the Food and Drug Administration (FDA) for the diagnosis of candidiasis, it associated with high false-positive, low sensitivity and

Test	Sensitivity (%)	Specificity (%)	Reference
Mannan and antimannan	58 and 59	93 and 83	[18]
(1,3)-ß-D-glucan	75–80	80	[19]
C. albicans germ tube antibody	76.2	80.3	[20]
T2Candida	91.1	99.4	[20]
Polymerase chain reaction	95	92	[19]
PNA-FISH[a]	98–100	99–100	[21]
MALDI-TOF MS[b]	91–100	100	[11]

[a]*Peptide nucleic acid fluorescent in situ hybridization.*
[b]*Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.*

Table 1.
Sensitivity and specificity of some methods used in diagnosis of invasive candidiasis as compared to conventional methods.

specificity results as shown in the **Table 1** above. This has limited its use for screening purposes [16, 22].

Enzyme-linked immunosorbent assay (ELISA) kits can be used to identify both mannan and anti-mannan antibodies however, this test is not recommended for identification of *Candida* species due poor specificity and sensitivity [23]. *C. albicans* germ tube antibody is an indirect immunofluorescence assay that detects antibodies against *C. albicans* germ tube and commercial kits for CAGTA assay include VirClia IgG Monotest and Vircell kit (Vircell, Spain). However, FDA has not yet approved CAGTA for use in clinical settings [24].

2.2 Molecular method

Molecular methods are more accurate and rapid in detecting *Candida* species. They have higher sensitivity and specificity as shown in **Table 1** above. Most molecular methods have the power to rapidly detect both primary and secondary antifungal resistance alleles, which may necessitate these methods to progressively replace conventional techniques which have reduced sensitivity and specificity as shown in **Table 1** above [15]. The D1/D2 region located in the larger ribosomal deoxyribonucleic (DNA) subunit and intervening transcribed spacers (ITS) 1 and 2 located between 18S, 5.8S, and 28S ribosomal ribonucleic acid (RNA) genes as shown in **Figure 1** below are useful markers for *Candida* species identification and phylogenetic studies [11, 15].

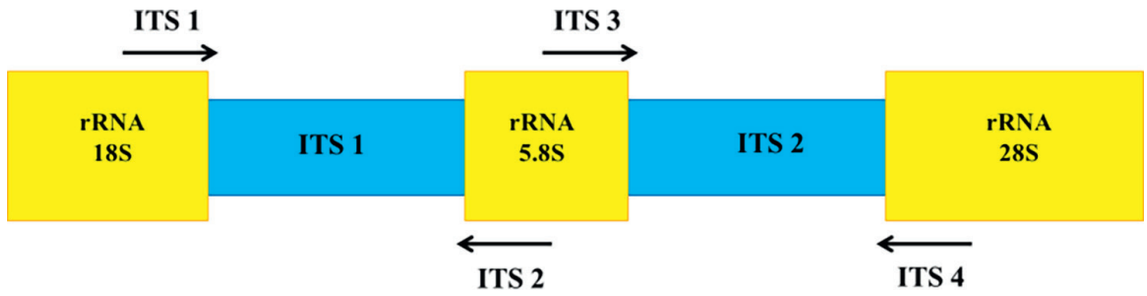

Figure 1.
Adopted from Chen et al., 2000, showing the non-coding internal transcribed spacer between the coding regions of 18S, 5.8S and 28S ribosomal RNA [25].

These regions contain sufficient sequence heterogeneity to provide differences at the species level [26].

Molecular techniques are categorized into two methods, i.e., polymerase chain reaction-based methods and non-polymerase chain reaction (PCR) based methods.

2.2.1 Polymerase chain reaction-based techniques

Polymerase chain reaction (PCR) is one of the most important molecular techniques used to detect *Candida* species, as it is fast and easy use [15].

2.2.1.1 Polymerase chain reaction (PCR)

PCR is based on the amplification of a small specific DNA target through multiple repeated cycles of temperature changes into multiple copies. The main PCR steps are denaturation of the template DNA into single strands (94–98°C), annealing of the primers to the target sequence (50–65°C), and elongation whereby DNA polymerase elongates a DNA complementary to each strand of the target (72°C) [27].

Various PCR techniques have been developed, such as real-time PCR, Restriction Fragment Length Polymorphism (RFLP) PCR technique, multiplex PCR, reverse transcriptase PCR and nested PCR [27, 28].

Real-time PCR can be used to quantify the PCR product during amplification. Moreover, it has advantages over the conventional PCR in that it does not require agarose gel electrophoresis to visualize the amplified products. In real-time PCR, the amplified product can be measured automatically after each cycle by a fluorometer [29].

Nested PCR is based on the amplification of DNA by using two sets of primers to improve its specificity and sensitivity. *Candida* DNA topoisomerase II genes have been used to adjust Nested PCR for identification of specific *Candida* species [30]. Reverse transcriptase PCR (RT-PCR) is based on the reverse transcription of ribonucleic acid (RNA) into complementary DNA (cDNA) using a reverse transcriptase enzyme. The cDNA can then be amplified by regular PCR [31].

Restriction Fragment Length Polymorphism (RFLP) PCR is an important technique to detect and identify strains of *Candida* species using portions of ribosomal DNA, such as the intervening transcribed spacers (ITS) region that are located in between the small and large ribosomal subunits, and the D1/D2 region of the large (26S) ribosomal subunit [12, 22, 32].

Multiplex PCR requires multiple different primers and specific probes labeled with different fluorophores in a single PCR tube to allow the identification of many different *Candida* species from the same sample. For instance, amplification of two DNA fragments from the ITS1 and ITS2 regions in combination with specific primers in a single PCR reaction is very accurate in *Candida* species speciation [33]. It is worth noting that multiplex PCR has the following advantages, has a high specificity and sensitivity of approximately 2 cells per, is rapid and easy to use, whole yeast cells may be employed directly in the PCR mixture, has the potential to discriminate specific *Candida* species in polyfungal infections to a maximum ratio of 1:10, and has a good reproducibility among different PCR thermal cyclers and within different laboratories [34]. In addition, commercial Multiplex qPCR kits for *Candida* detection including *Cand*ID/Plus (OLM diagnostics, UK) and Fungiplex *Candida* IVD (Bruker, Germany) are now available [17, 33].

2.2.1.2 Sequencing

Sanger sequencing is a first-generation sequencing technique developed by Sanger Frederick and it is based on chain-termination (Sanger *et al.*, 1977). Sanger sequencing has been used extensively for identification of many fungal pathogens [35]. The most commonly conserved regions in fungi are the ribosomal RNA genes including 5.8S, 18S and 28S and in between these are the ITS1 and ITS2 regions, non-coding regions, which vary in different species and sequencing of these regions supports rapid identification of different *Candida* species [15, 22]. Limitations of Sanger sequencing include high cost for whole genome sequencing and reduced accuracy when using only one copy for each strand [35].

Next generation sequencing (NGS) is accurate and rapid high throughput sequencing technique and is very vital in genome sequencing, fungal research, diagnostic purposes, outbreak monitoring [36]. Most of NGS platforms including the Ion Torrent PGM (Life Technologies), HiSeq, MiSeq and NextSeq (Illumina), 454 GS (Roche) and SOLiD System (Applied Biosystems) are based on sequencing by synthesis and have three main steps: template preparation, sequencing and imaging and data analysis [37, 38]. In addition to *Candida* species identification, NGS can be used for detecting genetic mutations associated with antifungal resistance [15]. As compared to Sanger sequencing, NGS is accurate and faster as massive DNA strands can be sequenced in parallel on a single run and a lesser amount of DNA is required. However, NGS reagents are expensive and the software requires technical expertise [38]. Nanopore sequencing is the fourth-generation DNA sequencing technology which is fairly cheap and uses short sequencing time with long sequencing reads [39]. Nanopore platforms like GridION™, PromethION™ and MinION™ are the latest portable and affordable NGS technologies with high genotyping accuracy [40].

Pyrosequencing is another PCR based technique which depends on the release of pyrophosphate when nucleotides are incorporated into the nucleic acid chain by DNA polymerase and produced pyrophosphate is then subsequently converted to Adenosine-5'triphosphate (ATP) by ATP sulfurylase, and that provides energy for luciferin oxidation by luciferase, which produces light that can be detected as a peak on the pyrogram [37]. Any unincorporated nucleotides are degraded by apyrase to allow iterative nucleotide addition into the nucleic acid chain and peak heights are associated with the number of the same nucleotides added to the nascent strand [41].

Pyrosequencing is a rapid and accurate molecular method for the detection of point mutations in any selected gene within short DNA fragments. It has been used widely for the identification and detection of antifungal drug resistance [42].

2.2.2 Non-polymerase chain reaction-based methods

These methods can facilitate rapid identification of *Candida* directly from candida culture broth without the need for DNA amplification. Non-PCR methods include peptide nucleic acid fluorescent *in situ* hybridisation (PNA-FISH) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). These methods have sensitivity and sensitivity of up 100% in detecting *Candida* species causing invasive diagnosis as shown in **Table 1** above. PNA-FISH is based on the rapid hybridization between synthetic oligonucleotide fluorescence-labeled probes and species-specific ribosomal RNA that can be detected via fluorescent microscopy [28]. PNA-FISH has been used to effectively identify *Candida* species with high

sensitivity and specificity directly from positive cultures, with final identification provided within 2.5 hours [12]. However, this test is very expensive and needs technical expertise [11].

MALDI-TOF MS is a method that uses mass spectrometry to identify the protein fingerprints of microorganisms that are compared with databases of reference spectra [43]. MALDI-TOF MS is able to accurately detect and identify *Candida* species in a timely manner with up 100% sensitivity and specificity as shown in **Table 1** above. However, high set up cost is the main limitation of this test include the high setup [44].

2.2.3 The internal transcribed spacer marker for Candida species identification and phylogenetics

The ITS region of ribosomal DNA (rDNA) is the most useful genetic marker for rapid and accurate molecular identification of *Candida* species and phylogenetic studies due to its region sequence variability among different species [15, 45–47]. The ITS 1 and ITS 2 are two vital non-coding regions composed of conservative and variable subregions outside and inside respectively [45]. The ITS1 fragment is positioned between the 18S and 5.8S ribosomal RNA genes while ITS2 fragment is positioned between 5.8S and 28S ribosomal RNA genes [48]. Furthermore, the amplicon sizes differ according to the target ITS1 region based on specific *Candida* species of interest [33, 49]. It is worth noting that ITS primer design, PCR amplification and sequencing has been made easy due to availability of several conserved sequences, frequent copies of the ribosomal operon and moderately limited length of ITS region [48].

3. Conclusions

Emergence of non albicans *Candida* species causing Candidiasis has highlighted importance of accurate *Candida* species identification. Laboratory diagnosis of Candidiasis is often based on conventional and biochemical identification of *Candida* species. However, these methods are labor intensive, time consuming and often do not permit sufficient specificity and sensitivity. Furthermore, conventional based identification of *Candida* species is affected by the variable nature of phenotypic characteristics. Molecular based methods are more proficient, rapid and easier diagnostic technologies for Candidiasis due to their increased sensitivity, specificity and accurate early detection of different *Candida* species. Early diagnosis allows clinicians to combat Candidiasis at an early stage through choice-specific and effective antifungal therapy, avoiding empirical management and development of resistance to antifungal drugs. From this review, it is expected that progress in use of molecular approaches will continue to have a positive impact on exploration of molecular epidemiology of *Candida* species and subsequently improve diagnosis and management of candidiasis.

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