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## Laboratory Isolation and Identification of *Candida* Species

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**Abstract:** The yeast *Candida* being the main cause of candidiasis is a commonly isolated pathogen from immunocompromised patients. Successfully identifying the species of *Candida* is important in the treatment and management of the disease. The trend in the resistance acquired by some species of *Candida* leads to the importance of identification to the species level. This may avoid prescription of antifungal drugs that may not be available to specific species, for instance *Candida krusei* is intrinsically resistant to fluconazole but *Candida parapsilosis* may be susceptible. There are many techniques that can be adopted by hospitals or independent diagnostic laboratories to aid in the identification of this yeast. This study reviews various methods of isolation and identification starting from the collection of specimen, transporting them to a diagnostic laboratory followed by staining, microscopy, automated blood culture systems, biochemical tests and molecular techniques that are used for the identification of *Candida* species. The cost, expertise, laboratory facilities and the population needs of each geographical region should be considered in adopting these techniques for the diagnosis of *Candida* infections.

**Key words:** Laboratory identification, *Candida*, candidiasis, molecular identification, PCR, cultures

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### INTRODUCTION

The study of infectious diseases caused by fungi greatly attributes the study of Pasteur and Koch with pathogenic bacteria. Following their study, in 1839, microbiologists Schonlein and Gruby studied *Trichophyton schoenleinii* whereas Langenbeck had reported on the causative agent for thrush which was *Candida albicans* (Wilson and Gisvold, 2004). Then, great concern over bacteriology overshadowed mycology for many years. However, the rise of incidences in mycoses had received serious attentions to medical mycology. The most suitable first-line antifungal regimen is still an unknown fact. The choice of drug selection depends on the physician's knowledge of a drug, drug availability, patient's condition, concomitant medications and cost (Gallagher *et al.*, 2005). Antifungal drugs, mainly those containing azole groups such as itraconazole, ketoconazole and fluconazole have been used in the treatment of initial and subsequent *Candida* infections. However, there have been reports showing the difficulties in complete eradication of this fungus from patients as they seem susceptible to the antifungal drugs used. The

reason could be due to the genetic differences among the fungal species or simply due to the overuse of azole drugs. Therefore, it is appropriate for physicians to have information on the type of *Candida* species before prescribing antifungal drugs to these patients. This study reviews laboratory methods that can be employed to identify *Candida* spp. in fungal infections.

### FUNGAL DISEASES

Fungal infections fall into four broad categories, i.e., the deep-seated systemic mycoses, cutaneous mycoses, subcutaneous mycoses and superficial mycoses. Systemic mycoses occur sporadically and are caused by heterogeneous groups of fungi. Fungal spores that are inhaled may cause histoplasmosis, blastomycosis, sporotrichosis, coccidioidomycosis, cryptococcosis and paracoccidioidomycosis (Brooks *et al.*, 2010). Cutaneous mycoses are infections of the keratinocytes of the epidermis and their appendages such as hair and nails. Most common are the dermatophytoses, caused by dermatophytes from the genera *Trichophyton*, *Microsporon* and *Epidermophyton* (Ichhpujani and

Bhatia, 2007). However, subcutaneous mycoses involve the skin, subcutaneous tissues and bones resulting from the embedment of saprophytic fungi in these regions of the body without being disseminated to the internal organs. The principle mycoses are mycetoma, chromoblastomycosis, sporotrichosis and rhinosporidiosis (Arora, 2004). Superficial mycoses are only surface infections of the skin, hair, nail and mucous membranes. They are caused by a variety of fungi such as dermatophytes, *Candida* spp., *Malassezia*, *Exophiala*, *Trichosporon*, *Piedraia* and many others.

Apart from the above categories of mycoses, opportunistic mycoses are another major concern among immunocompromised patients. An opportunistic organism causes disease at any instance when a person's body is in a compromised state. The overuse of antibacterial antibiotics, immunosuppressive agents, cytotoxins, irradiation and steroids lead to this new category of systemic mycoses. These patients have been deprived of immune resistance by the body's normal flora, thus develop opportunistic mycoses, for example candidiasis, aspergillosis, mucormycosis and others. *Candida albicans* is a common cause of opportunistic mycosis. Oral candidiasis is common among AIDS patients, poorly nourished patients and immunosuppressed patients. Incidences of oral thrush among school children between the age of 6 and 13 years old have been reported in Nigeria (Nneka and Ebele, 2005).

In Malaysia, *Candida* species have been isolated from women diagnosed with vaginal candidiasis from a teaching hospital and it was found that *Candida albicans* was the predominant species among the strains isolated. The other species were *C. glabrata*, *C. lusitaniae*, *C. famata*, *C. krusei* and *C. parapsilosis*. Re-infection from these yeasts varied among individuals either by identical, similar or different strains or species of *Candida* (Chong *et al.*, 2003). In another report, the most predominantly isolated species from two hospitals in Malaysia from the year 2004 to 2009 was *Candida tropicalis*, followed by *C. albicans*, *Candida parapsilosis*, *C. krusei*, *Candida rugosa*, *Candida dubliniensis* and *Candida glabrata* (Madhavan *et al.*, 2010). Other studies have reported that there is an increase in the number of disseminated candidiasis among acute leukaemia patients following chemotherapy (Cantu, 2005). Basetti *et al.* (2007) reported that the incidence of candidemia within a one-year period of study was 13% among hospitalized patients in two teaching hospitals in Italy. Due to the strain resistance towards amphotericin B, some of these patients were given fluconazole, caspofungin and voriconazole for re-infections. In other cases of acute leukaemia patients,

oral colonisation by *Candida* species was found in 90% of the patient population. Treatment with ketoconazole among these patients showed little effect and complete eradication was only seen in 9 out of 20 patients treated for 5 days (Rodu *et al.*, 2006). Related studies have shown that there were increased disseminated *Candida* infections among bone marrow transplant patients and neutropenic patients by *Candida krusei* since a century ago (Wingard *et al.*, 1991). The role of a diagnostic laboratory is important in the management of the fungal infections. The ability to identify the causative agent of the fungal infection to the genus, species or the strain, to a great extent, can determine the treatment for the infection and minimize treatment failure or recurrent infections.

### **SPECIMEN AND TRANSPORT**

All specimens should have a laboratory test requisition form each completely filled by the physician with the patient's name, age, sex, specimen source and patient's history (Tortora *et al.*, 2010). In order to recover yeast cells in specimen, adequate amount of specimen is necessary to perform desired tests. For example, 5 mL of cerebrospinal fluid is recommended as an optimum volume for laboratory diagnosis. In adults with bloodstream infections, 10 mL of blood should be drawn into each culture bottle. Generally, two aerobic and two anaerobic culture bottles are recommended (Brooks *et al.*, 2010). The laboratory manager or technicians should inform the physician if additional amount of specimen is required. Specimens obtained from skin, nail clippings and hair should be placed in an envelope, Petri dish or any other suitable containers and sealed properly. Specimens from mucous membranes can be inoculated in a medium or directly smeared on a clean slide using an inoculation loop or a swab. Slides are covered with cover slips and should be placed in an envelope or a slide box and sealed. In subcutaneous infections, scrapings, crusts, aspirated pus or tissue biopsies should be removed aseptically and placed into a sterile container. In systemic infections, specimens can be drawn from blood, cerebrospinal fluid or other areas directly into appropriate sterile vials containing blood culture medium (Chakravarthi and Haleagrahara, 2011). If there should be a delay in transporting the specimens to the diagnostic laboratory, then the specimens should be incubated at 35°C.

### **CULTURE MEDIUM**

The basic culture media used in isolating clinical *Candida* species are blood agar, Potato Dextrose Agar (PDA) or broth (PDB), Sabouraud brain heart infusion

agar, Sabouraud Dextrose Agar (SDA) or broth (SDB), Yeast Nitrogen Base (YNB) and Yeast Potato Dextrose (YPD) agar or broth. Lee's synthetic medium can be used for mycelial development and yeast formation for *Candida albicans* (Jatta *et al.*, 2009; Yang *et al.*, 2009). Other media that can be used as a selective or differential media are CHROMagar Candida (Madhavan *et al.*, 2009; Bernal *et al.*, 1996), CandiSelect4 (Gaschet *et al.*, 2008) and Pharmamedia (Slifkin, 2000). Pagano-levin agar can also be used as a differential medium but the presence of Triphenyltetrazolium Chloride (TTC) was found to inhibit the growth of some species of *Candida* (Yamane and Saitoh, 1985; Samaranyake *et al.*, 1987). Therefore, *Candida* Bromocresol Green Agar (BCG Agar) is now used as an alternative as TTC was replaced with a non-toxic inhibitor, bromocresol blue. Since *C. albicans* from *C. dubliniensis* are closely related species which can be difficult to differentiate phenotypically, cultivation on Bird-seed agar was found to be a fast, reliable and sensitive method to discriminate these two species (Pasligh *et al.*, 2010). Agar plates provide a large surface area to perform the streaking method. This method would enable the identification of any contaminants or transient normal flora. It was also recommended that the least selective medium should be inoculated first with the most selective and inhibitory media last. This would prevent carry-over inhibition from one medium to another (Nye *et al.*, 2006). Sabouraud's dextrose agar is also available as Mycosel which contains chloramphenicol and Mycobiocin inhibitory mould agar to inhibit bacterial growth. Sterile body fluids and tissues samples are recommended to be cultured on Trypticase soy blood agar and incubated between 35 to 37°C for 72 to 96 h. Long term storage for few months can be done using agar slants (Chakraborty *et al.*, 2005).

### MICROSCOPY

The microscope is the best tool for a microbiologist. Distinct features of yeasts can be identified by observing their morphology. Microscopes can be used for fast identification and detection of possible yeasts in a clinical sample. Specimens from exudates, sputum, urine and cerebrospinal fluid can be viewed under reduced-light brightfield microscope or phase-contrast microscope (Aslanzadeh and Roberts, 1991). Presumptive identification of *C. albicans* is done with the germ tube test. In this method, the clinical sample is incubated in human or animal serum for 2 to 3 h at 30 to 37°C. If *C. albicans* is present, short, slender, tubelike structures (germ tube) can be observed under the microscope (Mackenzie, 1962). Reports of other species of *Candida*

such as *C. tropicalis* and *C. parapsilosis* were also found to produce similar structures (Campbell *et al.*, 1998; Freydiere and Guinet, 1997; Lipperheide *et al.*, 1993; Perry *et al.*, 1990). However, stains can be used as an aid to view yeasts in specimens. Potassium hydroxide solution (10-20% KOH and 10% glycerine) is the most commonly used stain to detect fungi or yeast cells. KOH digests keratin and glycerine prevents degradation of yeast. Lactophenol cotton blue can also be used together with KOH for better observation of yeast under brightfield microscope. Gram and Giemsa stains are useful in staining yeast cells because of their small size (3-4 µm). Calcofluor white, a colourless dye can be used to detect fungal elements even in frozen and paraffin-embedded tissue sections (Aslanzadeh and Roberts, 1991). The advantage of using this dye is that stained specimens can be viewed immediately using an improvised and economical fluorescent microscope with 25 watt halogen lamp rather than epifluorescent or mercury vapour lamp. Periodic acid Schiff can be also used to detect fungi in tissues but may take several h to perform the test. However, methenamine silver is known to be the best method to detect fungi in histology. Fungal elements would be seen as black against a red or green background. Apart from these, stains used in cytology and pathology laboratories are hematoxylin-eosin and papanicolaou which are found to be less effective than methenamine silver (Chakraborty *et al.*, 2005).

### AUTOMATED BLOOD CULTURE SYSTEM

Automated culturing systems detect microbial growth automatically by monitoring the CO<sub>2</sub> production released from the metabolic activity of the microbial cells. The advantages of this system are it is more sensitive than manual systems and does not require manual inspection or examination of the culture (Ryan and Murray, 1993; Han, 2006). There are various BACTEC systems that are used in hospital laboratories to detect many microorganisms. Several systems can be used for yeast identification in blood such as BacT/Alert standard, BACTEC 9240 standard, BacT/Alert FAN, BACTEC fungal medium and BACTEC Plus Anaerobic/F bottles (Han, 2006). Blood samples are inoculated directly into 2 bottles with a maximum of 10 mL in each bottle. Detection of *Candida* cultures may differ from one species to another, example the mean time for positive detection of *Candida albicans* is between 35.3 to 85.8 h where as for *Candida glabrata* is between 80 to 154 h (Fernandez *et al.*, 2009). Yeasts cultures are usually held for 21 days and blood cultures for about 5 days.

## BIOCHEMICAL TESTS

Biochemical tests are also routinely done following the initial phenotypic identification of the cultures on agar media and microscopy. Tests using single enzyme are able to detect the presence or absence of an enzyme or a biochemical reaction within seconds to minutes. These tests are economical, rapid and simple to perform (Aslanzadeh, 2006). Various *Candida* species can be detected by observing the changes in the indicator colour when the yeast cultures utilize 1% carbohydrates such as glucose, maltose, sucrose, trehalose and raffinose. These tests are now available as commercial kits such as API 20C, API 32C or RapID Yeast Plus systems. Other than carbohydrates, hydrolysis of 1% fatty acid ester, 0.05% aryl-substituted glycosides, 0.3% urea and 0.01% arylamide substrates can be detected with RapID Yeast Plus system. The resulting colours at the end of the incubation period are coded and compared with the RapID Yeast Plus Differential Chart to identify the species. This method is currently the fastest commercial method for the identification of yeasts which requires a 4 h incubation period only. However, the identification of *Candida dubliniensis* was found better with API 32C than Vitek-2 YST system (Cardenes-Perera *et al.*, 2004). API 32C was also useful in differentiating *C. albicans* from *C. dubliniensis* as this two species are phenotypically alike. API 32C is based on the assimilation of various carbohydrates and Vitek-2 YST system is based on the detection of enzymes in the yeast species. It was reported that Vitek-2 YST system is an automated new colorimetric card system which could correctly identify *Candida* species in 18 h which is faster than API 20C and API 32C (Loiez *et al.*, 2006). However, in another report, there was a necessity for additional tests with CHROMagar and API 32C to verify the results of Vitek-2 YST system (Pryce *et al.*, 2006). It was also suggested that conventional diagnostic methods are not reliable to identify newly emerging *Candida* pathogens such as *C. haemulonii* (Ruan *et al.*, 2009) and *C. kefyr* (Gomez-Lopez *et al.*, 2010), as they were often mis-identified as other species of *Candida*. In their report, it was also stated that although the VITEK 2 system was found better compared to VITEK 1 and API 32C, it still could not be used to differentiate *C. haemulonii* from its sibling species *C. pseudohaemulonii*.

## NUCLEIC ACID-BASED IDENTIFICATION

Nucleic acid sequence of a gene is an important property as it carries the identity of an organism.

Molecular methods are based on the detection of the nucleic acid sequence of a gene specific to an organism. In probe-based identification, the organism's single stranded DNA or RNA would bind to a complementary sequence and form hybrid which is double stranded. Identification of specific organisms can be done from the clinical specimen, culture, formalin-fixed or paraffin-embedded tissues (Hong, 2006). The use of rRNAs is essential for probe-based detection of species as its nucleotide sequence is well conserved within a species and varies between species. Therefore, it is used as a target for species identification. Sequencing the amplified regions of Internal Transcriber Spacer regions (ITS) of the rRNA gene for *Candida* gained success in species identification (White *et al.*, 1990). The early discoveries were in *C. dubliniensis* (Sullivan *et al.*, 1995), *C. orthopsilosis* and *C. metapsilosis* (Tavanti *et al.*, 2005) using PCR methods. In Denmark, differentiation between *C. orthopsilosis* and *C. metapsilosis* was performed using a newly described RFLP method using SADH (secondary dehydrogenase-encoding gene), 26S rRNA (D1/D2) and ITS1-ITS2 regions (Mirhendi *et al.*, 2010). In another study, molecular identification using ITS1-ITS4 fungal primers were able to identify *C. orthopsilosis* which was previously identified as *C. parapsilosis* with API 20C test kit (Yong *et al.*, 2006). Very recently, a rapid real-time PCR method was also developed to distinguish *C. metapsilosis*, *C. orthopsilosis* and *C. parapsilosis*. Different melting curve used was able to identify the three species mentioned above. Hays *et al.* (2011) and Gomez-Lopez *et al.* (2010) reported that a strain which was identified as *C. kefyr* with biochemical test method was discovered to be *C. sphaerica* by ITS sequencing. In another report, strains of *C. haemulonii* was mis-identified as *C. sake*, *Pichia ohmeri* and other *Candida* spp. by biochemical test kits (Ruan *et al.*, 2009). However, molecular methods revealed their species identity. A multiplex PCR method used in another study was able to differentiate *C. nivariensis* and *C. bracarensis* which are phenotypically identical to *C. glabrata* (Romeo *et al.*, 2009).

Affirm DNA probe system is a diagnostic test that can detect and differentiates causative agents of vaginitis, mainly *Candida*, *Gardnerella* and *Trichomonas* (Hong, 2006). This system uses two distinct single stranded DNA that is complementary to unique sequences of target organisms. These probes are known as capture probe and colour development probe. The Affirm VPIII Microbial Identification Test yields positive results only for symptomatic vaginitis based on the organisms' cell count. This is very useful in terms of detecting symptomatic vaginitis or vaginosis and

providing appropriate treatment. PNA FISH probe is a new diagnostic technique using fluorescence in situ hybridization using Peptide-Nucleic Acid (PNA) probes. Peptide nucleic acids are synthetic compounds containing nucleotide bases attached to a peptide backbone which targets the rRNA. PNA FISH for *Candida albicans* has been approved by FDA and is commercially available. Pulsed-Field Gel Electrophoresis (PFGE) which was developed in early 1980s is currently used for strain characterization, to understand the evolution of antimicrobial resistance and epidemiological analyses. The source of transmission can be identified especially in nosocomial infections and to curtail disease outbreaks using this technique (Wu and Della-Latta, 2006). PFGE has been used in genotyping *Candida* spp., with or without the use of restriction enzyme digest in its protocol (Espinel-Ingroff *et al.*, 1999). The identity of the infective strains in a patient with recurrent *Candida* infections over a period of time is also possible with this technique (Bennett *et al.*, 2004). A combination of molecular typing methods with high and low discriminatory power is necessary to provide optimal source tracking especially for nosocomial infections of *Candida* spp. High discrimination allows detection of strain variants and low discrimination allows the isogene determination of organisms (Lopez-Ribot *et al.*, 2000). Therefore, to fulfil both these requirements, *Candida* specific DiversiLab Kit and DiversiLab Yeast Kit can be used (Frye and Healy, 2006).

There are many other techniques that can be used for strain typing or identification such as Ca3 genetic fingerprinting for *Candida albicans* (Martinez *et al.*, 2002; Pujol *et al.*, 2002, 2004), Multilocus Sequence Typing (MLST) (Bougnoux *et al.*, 2006; Chen *et al.*, 2006; Viviani *et al.*, 2006) Multilocus Enzyme Electrophoresis (MLEE) (Arnavielhe *et al.*, 2000; Boldo *et al.*, 2003), Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD) analysis (Chong *et al.*, 2007; Lephart *et al.*, 2004; Roilides *et al.*, 2003; Samaranyake *et al.*, 2003), microsatellite-PCR (Lopes *et al.*, 2007; Correia *et al.*, 2004; Dalle *et al.*, 2003) and Amplified Fragment Length Polymorphism (AFLP) (Lopes *et al.*, 2007). Reverse transcription PCR (RT-PCR) analysis can be also used for the identification of mutations and gene regulations in *Candida* species (Francisca *et al.*, 2011; Yong *et al.*, 2006; Looi *et al.*, 2005). However, the use of above stated methods depend on the aim of the identification. Routine identification in a laboratory using molecular techniques remains challenging as some of these techniques may be costly, require trained personnel and still lack sufficient species-specific primers. Therefore, the selection of methods to identify an organism is not

solely based on the effectiveness of the methods but also the availability and affordability of them.

## CONCLUSION

A well trained mycologist is still an important criterion to access the results of various diagnostic techniques used in a laboratory. Therefore, offering a comprehensive diagnostic service not only requires resources but also up-to-date training for the laboratory staff to encounter the ever evolving diagnostic methods.

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