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Review Article

Laboratory approach for diagnosis of candidiasis through ages

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A B S T R A C T

Fungi, once considered non-pathogenic microbiological curiosities have emerged as an important cause of community acquired and health care associated infections. Among various pathogenic species of fungi, *Candida* is the most prominent cause of fungal infections. Although *C. albicans* is the most prevalent species involved in infections, the shift towards non-*albicans Candida* (NAC) spp. is documented in recent studies. Ironically, the clinical manifestation caused by NAC spp. is indistinguishable from those caused by *C. albicans* but they differ in their susceptibility to antifungal agents and often demonstrate low sensitivity to commonly used antifungal drugs. Due to non-specific clinical presentation, diagnosis of disseminated candidiasis is difficult as compared to mucocutaneous candidiasis. Prompt and accurate identification of *Candida* species is very essential for effective therapeutic outcome. Conventional methods for the diagnosis of candidiasis are less sensitive and time consuming, hence, immunodiagnostic and molecular techniques can be recommended for early and specific diagnosis.

Keywords

Disseminated candidiasis; molecular techniques; mucocutaneous candidiasis; polymerase chain reaction.

Introduction

Fungi, once considered non-pathogenic microbiological curiosities have emerged as an important cause of community acquired and health care associated infections (Chakrabarti *et al*., 2000). Factors like HIV/AIDS, treatment with broad spectrum antibiotics and immunosuppressive drugs increase the vulnerability to fungal infections (Deorukhkar *et al*., 2012 a). Among various pathogenic species of fungi, *Candida* is the most prominent cause of fungal infections (Sullivan *et al*., 1996).

Although a part of normal microbiota, *Candida* is capable of causing various clinical manifestations ranging from mucocutaneous overgrowth to disseminated infections like candidemia (Eggimann *et al*., 2003). Only a few decades back, the role of *Candida* in establishment and progression of infection was considered to be passive, and organic weakness or an immunocompromised status of the host was considered as the vital mechanism responsible for candidiasis (Sardi *et al*., 2013). Therefore

candidiasis was called the "disease of diseased". Recently this concept has changed and it is established that *Candida* can actively participate in the pathophysiology of the disease progression by using mechanisms of aggression called virulence factors (Tamura et al. 2007; Sardi *et al*., 2013).

Virulence factors like tissue adhesion, phenotypic switching, biofilm formation and production of extracellular hydrolytic enzymes play an important role in colonization and invasion of host tissues (Sachin et al., 2012; Deorukhkar and Saini, 2013a; Deorukhkar and Saini, 2013b).

The genus *Candida* is composed of heterogeneous group of organisms and more than 17 different *Candida* spp. are implicated in human infections (Pfaller *et al*., 2007). Although *C. albicans* is the most prevalent species involved in infections, the shift towards non-*albicans Candida* (NAC) spp. is documented in recent studies (Enoch et al.,2006; Deorukhkar *et al*., 2012 a ; Deorukhkar and Saini, 2012 b; Deorukhkar and Saini 2013 c) . The change in the trends of candidiasis can be attributed to various factors like severe immunosuppression or illness, prematurity, exposure to broad spectrum antibiotics and empirical use of antimycotics.

Ironically, the clinical manifestation caused by NAC spp. is indistinguishable from those caused by *C. albicans* but they differ in their susceptibility to antifungal agents and often demonstrate low sensitivity to commonly used antifungal drugs (Johnson et al., 1995; Deorukhkar *et al*., 2012 a). Therefore identification of the infecting *Candida* to the species level is of utmost importance for clinical microbiological services for prediction of likely drug susceptibility and to guide treatment. In the present study, the literature on conventional and molecular techniques for diagnosis of candidiasis is reviewed.

Laboratory diagnosis of candidiasis

Like for any other infections, the laboratory diagnosis of candidiasis depends on the infection caused by it. As discussed earlier *Candida* is capable of causing a wide range of clinical manifestations. Figure 1 summarizes various clinical manifestations of *Candida* spp. This review is focused on the laboratory diagnosis of mucocutaneous and disseminated candidiasis.

Mucocutaneous candidiasis.

Laboratory diagnosis of this form of candidiasis involves.
Direct demonstration of fungal elements from clinical specimens.
Isolation of *Candida* in culture and species identification.

Direct examination

The direct examination of clinical specimens is done by wet mount preparation. Potassium hydroxide (KOH) facilitates the demonstration of fungal elements. The percentage of KOH varies from 10 to 40% depending on the nature of specimens (Segal and Elad 2005). KOH digests proteinaceous debris in the specimens and makes the visibility of fungal elements clear.

Addition of 36% dimethyl sulfoxide (DMSO) to KOH helps in rapid clearing of proteinceous debris and eliminates the requirement of heating in KOH

preparation before microscopic examination. Addition of Parker's ink or the lacto- phenol cotton blue also benefit the demonstration of fungal elements (Segal and Elad, 2005).

Of many modifications of KOH mount preparation, addition of calcofluor white (CW) allows the rapid recognition of fungal components. CW is a whitening agent used in textile and paper industry (Segal and Elad, 2005). It binds to chitin and cellulose in fungal cell wall and fluoresces on excitation by long wave ultraviolet (UV) rays or short-wave visible light. CW staining has become very popular due to its greater sensitivity as compared to other conventional stains (Aslanzadeh and Roberts, 1991). It can be also subsequently used with Gomori's methenamine silver (GMS) or periodic acid-schiff (PAS) stains (Segal and Elad, 2005). The requirement of fluorescent microscope with proper UV source limits its use in many laboratories.

The fungal elements like budding yeasts, pseudohyphae and or hyphae can be demonstrated by wet mount preparation and can be used as an indication of candidiasis. However, species identification requires isolation and biochemical or physiological characterization (Segal and Elad 2005).

Specimens from vulvovaginal candidiasis (VVC) and oropharyngeal candidiasis (OPC) generally do not require treatment with keratinolytic substances like KOH. For wet mount preparation, saline can be used. The addition of LPCB enhances the visibility of the fungal elements. The fixed smear can be stained by Gram staining, Giemsa or methylene blue.

In case of VVC and OPC, the demonstration of pseudohyphae along with yeast cells (Figure 2) is an important diagnostic feature to distinguish infection from normal colonization (Segal and Elad, 2005). In case of *C. glabrata*, an important cause of VVC, the diagnosis cannot be relied only on microscopic examination as this species of *Candida* is haploid and do not produce hyphae or pseudohyphae in-vitro (Deorukhkar and Saini, 2014).

Direct microscopic examination is a cost effective, rapid method for diagnosis of candidiasis. It requires less expertise. However it should be kept in mind as *Candida* spp. is a commensal of oral cavity and vagina, both its demonstration in direct smear and isolation in culture are important for establishment of diagnosis.

Culture

Candida spp. being non- fastidious organism, readily grows on most laboratory media used for the isolation of fungus. Sabouraud dextrose agar (SDA) is the most frequently used media for primary isolation of *Candida* spp (Odds, 1991). It permits the growth of *Candida* and suppresses the growth of many but not all bacteria due to its low pH.

On SDA *Candida* produces creamy, smooth, pasty and convex colonies which may become wrinkled on further incubation (Figure 3). SDA is not a selective and differential medium (Samaranayake *et al*., 1987). Supplementation of SDA with antibiotics (Chloramphenicol, gentamicin and/or tetracycline) and cycloheximide (actidione) makes the medium selective. Antibiotics prevent the growth of bacteria, whereas cycloheximide avoids saprotrophic fungal contamination (Odds, 1991). As some strains of *C. krusei*, *C.*

tropicalis and *C. parapsilosis* are sensitive to cycloheximide, SDA with cycloheximide is not recommended as single isolation media (Segal and Elad, 2005).

The culture medium can be incubated at 28^0C or/and at 37^0C. *Candida* colonies appear on medium within 24 to 72 hours. Some species may require more than 3 days to appear on culture medium (Segal and Elad, 2005).

The selection of media for the identification of *Candida* spp. from clinical specimens harboring a mixture of yeast spp. is of critical importance (Coleman *et al*., 1993). Varieties of differential media are available for speciation of *Candida*. These include Pagano-Levin agar (Figure 4), phosphomolybdate agar, Nickerson's medium (Costa et al., 1964) and chromogenic medium (Figure 5) (Odds and Bernaerts, 1994).

Chromogenic media like CHROM agar *Candida* media, *Candida* ID medium and Candiselect 4 medium are used for rapid identification of *Candida* (Ghelardi *et al*., 2008). These media contains chromogenic substrates that react with enzymes secreted by yeast cells, resulting in various pigmentations. These enzymes are species specific and allow species identification on the basis of colony color and characteristics (Horvath *et al*., 2003).

CHROMagar *Candida* (Figure 5) is widely used for primary isolation and differentiation medium for clinical specimens likely to contain yeasts. It also acts as differential medium for identification of yeasts isolated on other media (Odds and Bernaerts, 1994). Although chromogenic media are more expensive, the use of these medium reduces the time required for identification of *Candida* as compared to other conventional methods.

Species identification

Speciation of *Candida* is done on the basis of colony characteristics, microscopic morphology, physiological or biochemical characteristics. Sero-diagnostic tests though available are seldom performed for superficial and mucocutaneous candidiasis.

Germ tube test

In diagnostic mycology the basic work up for yeast identification starts with germ tube test. Germ tube formation was first reported by Reynolds and Braude and hence the germ tube test is also known as "Reynolds-Braude Phenomenon" (Deorukhkar *et al*., 2012 c).

This is a rapid method for identifying *C. albicans* and *C. dubliniensi*s by its ability to produce short, slender, tube like structures called germ tubes when it is incubated in serum at 37^0C for 2 hours.

Due to the time required to prepare human serum and inherent safety problems concerned with its use, many clinical microbiological laboratories have started using non-human serum media for testing germ tube production. These include egg white, saliva, tissue culture medium, sheep serum, trypticase soya broth and various peptone media. Trypticase soya broth is found to be more stable, effective and safe than other media for production of germ tube (Deorukhkar et al., 2012 c).

In this test the observer must be able to differentiate between germ tube and

pseudohyphae. The elongated daughter cells from the mother cell without constriction at their origin are referred to as germ tubes (Figure 6) whereas constriction at the origin of mother cells is called pseudohyphae (Kim et al., 2002).
A criterion for germ tube positivity is observation of minimum five germ tube in entire wet mount preparation. Negative results are confirmed by examining at least 10 high power fields for the presence of germ tubes (Deorukhkar *et al*., 2012 c).

Physiological/biochemical characterization.

Species of *Candida* can be characterized by the patterns of their use of specific carbohydrates. *Candida* spp. metabolizes carbohydrates both aerobically (assimilation) and anaerobically (fermentation). Yeasts possessing the ability to ferment a given carbohydrate also assimilate it, but not necessarily vice versa (Segal and Elad, 2005).

The biochemical identification of *Candida* spp. is based on assimilation and fermentation of carbohydrates. The classical assimilation test developed by Wickerham and Burton was further replaced by simpler and quicker auxanographic method (Hazen and Howell, 2003).

Various modifications (manual and automated) of carbohydrate assimilation are now available (Figure 7). These include API-YI (yeast indent) system/ API-20 C system, Uni-Yeast-Tek system (UYT), ID 32 C system, Yeast-Biochemical Card (YBC), RapID Yeast Plus system, FUNGICHROM1and VITEK system. Though these commercial systems are costly they have several advantages like rapid identification, require no or less supplemental tests and have less subjective errors in the interpretation of results. VITEK 2 ID-YST system is widely used for rapid identification and susceptibility testing of yeast and yeast like organisms (Graf *et al*., 2000).

Fermentation tests are used to supplement carbohydrate assimilation test results. These tests are more difficult to perform and are prone to variation (Segal and Elad, 2005). Since fermentation tests are less reliable, most of the commercial Kit systems are based on assimilations tests (Odds, 1988).

Other conventional methods for speciation of *Candida*.

The formation of true hyphae, pseudohyphae, chlamydospores and arthroconidia also aids identification of *Candida* spp. For this purpose nutritionally deficient media like corn meal tween-80 agar, rice starch agar and rice starch tween agar are used. These nutritionally deficient media suppress the vegetative growth and promote sporulation.

Urease test can be used for identification of *C. krusei*, *C. lipolytica* and *C. humicola* (Dolan, 1971). Methyl blue SDA and Staib agar (niger seed agar) can be used for distinguishing of *C. albicans* and *C. dubliniensis*, which shares many phenotypic properties (Sullivan and Coleman, 1998; McCullough *et al*., 1999).

Serological and molecular methods.

The different species of *Candida* can be identified by using species-specific antisera. However, serological identification is not a standard routine procedure for laboratory diagnosis of candidiasis.

Molecular diagnosis though possible, is not done on a routine basis for the diagnosis of mucocutaneous candidiasis. Molecular diagnostic techniques include restriction fragment length polymorphism (RFLP) analysis, southern hybridization analysis, tRNA profile analysis and polymerase chain reaction (PCR) technology (Sullivan *et al*., 1996).

Disseminated Candidiasis

As compared to superficial and mucocutaneous candidiasis the clinical presentations of disseminated candidiasis (DC) are non-specific and complicated. Therefore the rapid and precise laboratory diagnosis of DC is crucial not only for species identification but also for timely institution of appropriate antifungal treatment (Ellepola and Morrison, 2005; Pappas et al., 2009). The laboratory diagnosis of DC involves the use of immunodiagnostic and other non cultural methods in addition to culture. The varied nature of clinical forms makes the specimen collection difficult in DC. In most cases, invasive procedures are necessary for specimen collection (Segal and Elad, 2005).

Direct examination

In DC direct demonstration of fungal elements is done by wet and fixed mounts. Like in mucocutaneous candidiasis, CW aids in increased demonstration of fungal elements. Histological specimens are stained with PAS or GMS. Immunohistochemical techniques are reported to have good sensitivity and specificity (Segal and Elad, 2005).

Culture

In DC as compared to other clinical specimens blood culture is easy to obtain. Blood culture is the gold standard for the diagnosis of candidemia. However, traditional methods of blood cultures lacks sensitivity and may require several days to become positive (Ahmad and Khan, 2012). Several advances in blood culture techniques have improved the detection of candidemia (Ellepola and Morrison, 2005). These include the development of lysis-centrifugation tubes and automated monitoring of blood culture bottles. The lysis centrifugation system increases the yield of *Candida* spp. recovered from blood by using a detergent to release fungi trapped within host phagocytic cells. The resultant sediment is plated onto five different agar media (Archibald *et al*., 2000; Procop *et al*., 2000). This method reduces the time between inoculation and detection of growth. This system is expensive and labor intensive for routine use (Ellepola and Morrison, 2005).

The single most important improvement in this aspect is the institution of automated blood culturing systems with continuous growth monitoring. Either colorimetric (BacT/ALERT 3D, Organon Te Knika Corp., Durham, NC) or fluorescent (BACTEC 9240, Becton Dickinson, USA) monitoring can now be conducted at approximately 10 min intervals. Blood culture systems using continuous manometric monitoring also exists (ESP, Difco, Detriot, Mich and O.A.S.I.S., Unipath, Inc) (Ellepola and Morrison, 2005).

CSF, tissue biopsies or bronchial washing are examples of specimens obtained from sterile sites. *Candida* isolation rate is low in these specimens. SDA without cycloheximide is used for inoculation of these specimens from mucocutaneous candidiasis.

In case of urine samples, colony counts are important to differentiate between colonization or contamination and infection. Positive urine culture, in the absence of indwelling urinary catheter, which yield >1x10^4 CFU/ml is considered as an indication of significant candiduria (Ellepola and Morrison, 2005).

Phenotypic species identification

It is similar to that of mucocutaneous candidiasis and is based on differences in microscopic and microscopic morphology and on physiological and biochemical characteristics of the *Candida* spp.

Immunodiagnosis

Immunodiagnosis of DC involves, Detection of antibodies, primarily in serum and Detection of antigens in body fluids.

Antibody detection

The clinical utility of antibody detection for diagnosis of DC is limited because of 2 main reasons (Ellepola and Morrison, 2005). False negative results in immunocompromised patients, where there is low or undetectable levels of antibodies.

False positive results in patients with superficial colonization

Currently two tests are available for antibody detection. This includes an ELISA based test for detection of anti-mannan antibodies (Platelia *Candida* antibody test, Bio-Rad Laboratories, France) and indirect immunofluorescence assay for detection of antibodies against *C. albicans* germ tube (CAGTA) (C. albicans IFA IgG; Virvell Laboratories, Spain) (Ahmad and Khan, 2012). The sensitivity and specificity of anti-mannan antibody test when used alone was reported to be 53% and 94% respectively whereas when this test was performed in combination with antigen detection the values were changed to 80% and 93%. Therefore it can be suggested that a combination of both tests should be for diagnosis of DC (Ellepola and Morrison, 2005). The overall sensitivity and specificity of CAGTA have been reported to vary from 77% to 89% and 91% to 100% respectively (Zaragoza *et al*., 2009).

Antigen detection

In DC, detection of Candida antigens in serum and other body fluids is an important diagnostic tool, particularly in immunocompromised patients where antibody production can be variable or nonexistent (Walsh and Chanock, 1997). Mannan is a major cell wall component of *Candida* and it accounts for upto 7% of total dry weight of cell and is released in blood circulation during infection (Ahmad and Khan, 2012). It is resistant to heat, proteinase and acidic pH (Reiss and Morrison, 1993). Dissociation of antigen-antibody complexes is very necessary for detection of mannan in the serum since these immune complexes masks antigenic sites and reduces the sensitivity of test.

The immune complexes are dissociated by boiling in the presence of EDTA or by treatment with enzymes like pronase (Ellepola and Morrison, 2005). Mannan can be detected in serum and other body fluids by a number of serological reactions like Enzyme linked immunosorbent assay (ELISA), radioimmunoassay (RIA), latex agglutination (LA) and reverse passive agglutination test (RPLA) (Bougnoux *et al*., 1990; Lemieux *et al*., 1990; Reiss and Morrison, 1993).

Figure.1 Clinical manifestations of candidiasis

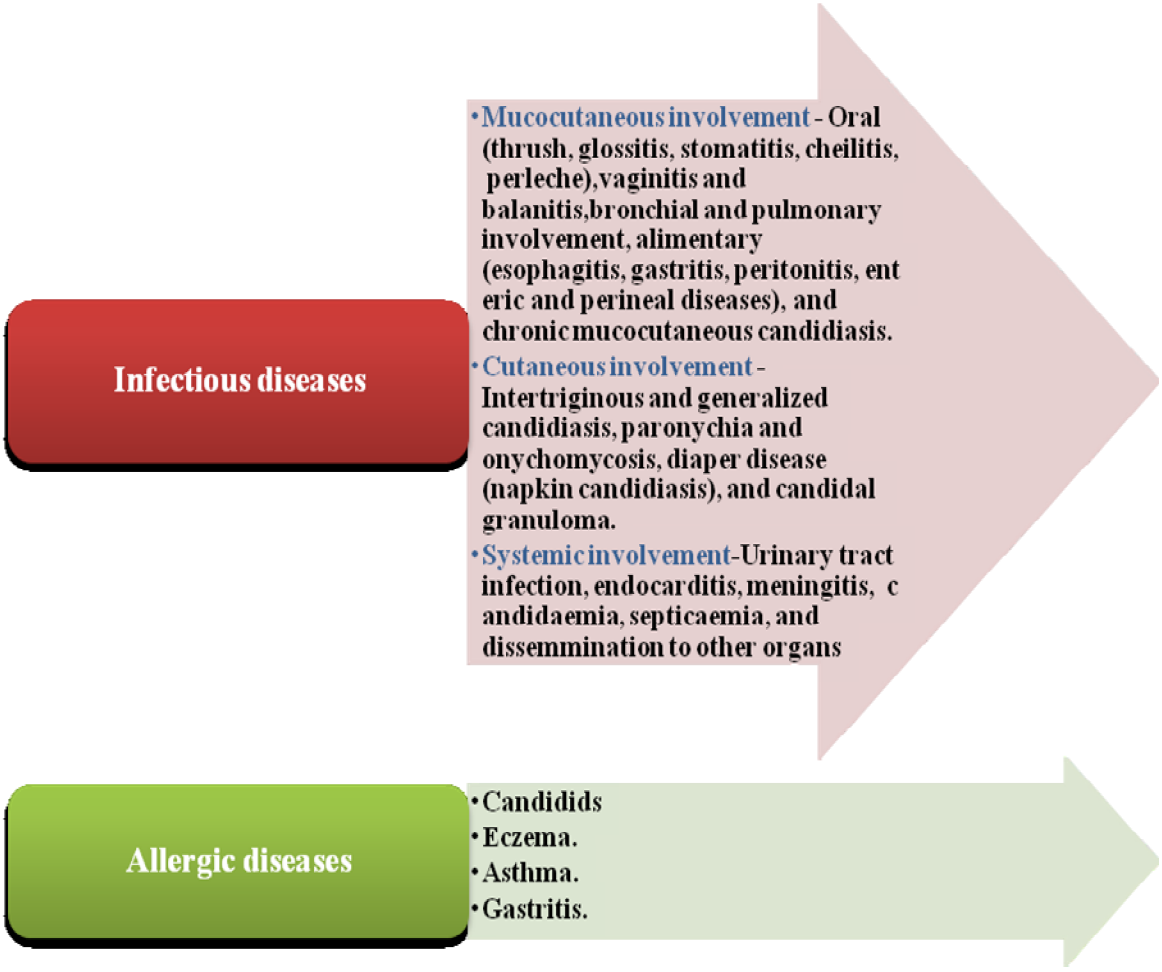


Figure.2 Gram stained smear showing Gram positive yeast cells with pseudohyphae.

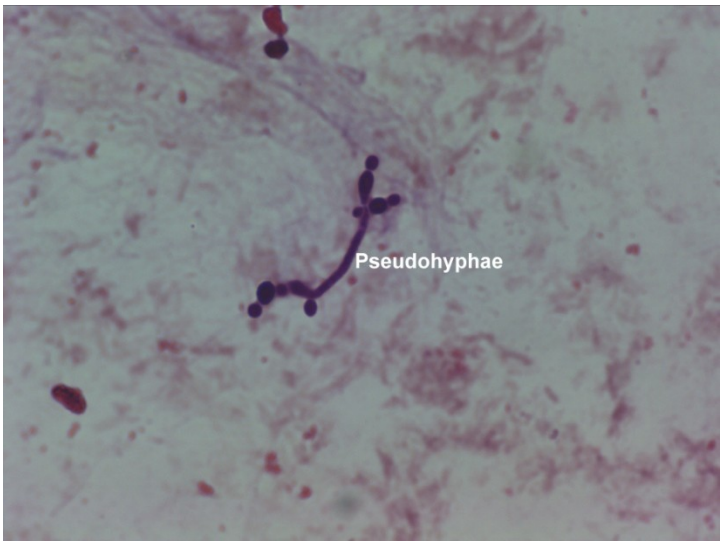


Figure.3 Colonies of *Candida* spp. on SDA

Figure.4 Different morphotypes of *Candida albicans* on Pagano-Levin agar

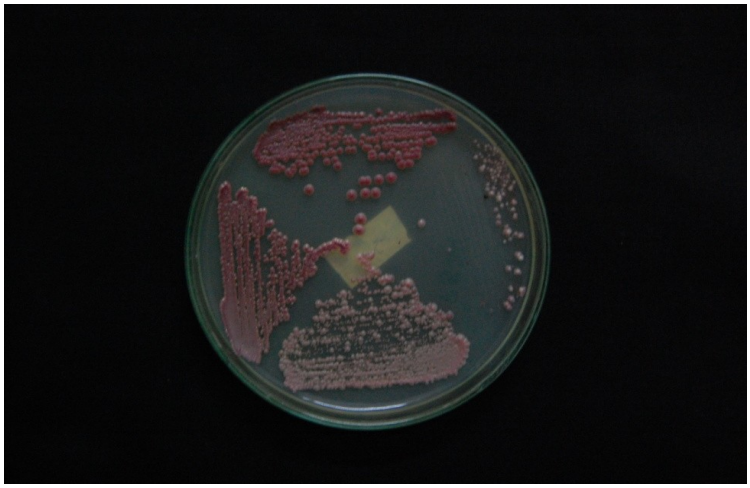


Figure.5 Differentiation of *Candida* spp. on CHROMagar

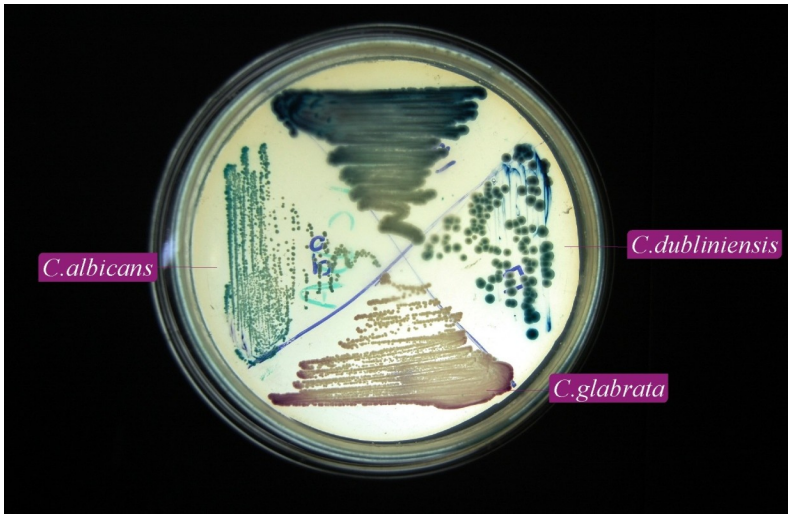


Figure.6 Germ tube test.

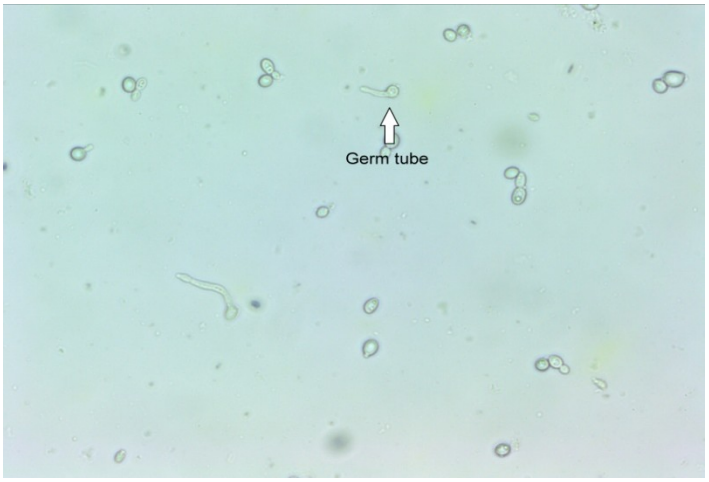
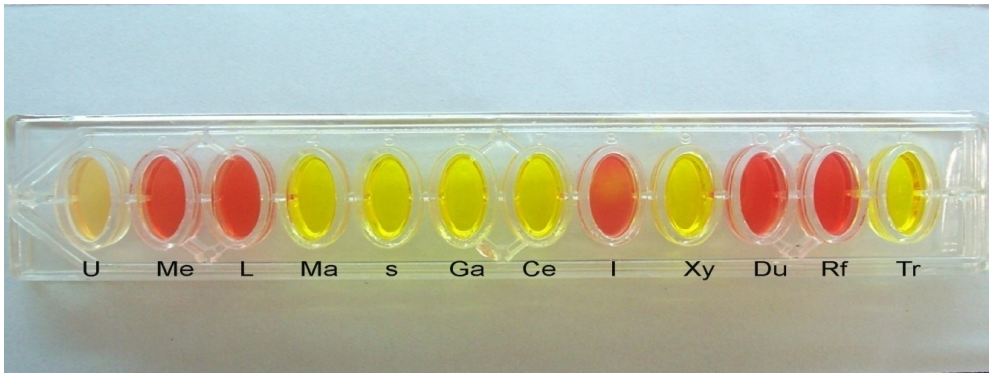


Figure.7 Hi-Candida identification kit showing carbohydrate assimilation reactions of *Candida albicans*



The platelia *Candida* Ag test (Bio-Rad Laboratories, Marnes-la-Coquette, France), a sandwich ELISA detects the presence of α-linked oligomannose residues (α-Man) released from *Candida* cells in serum and other body fluids (Ellepola and Morrison, 2005). The overall sensitivity and specificity of this test is reported to be 58% and 93% respectively (Ahmed and Khan, 2012). As mannan is rapidly cleared from the circulation, this test requires multiple serum sampling.

Several factors like frequency of sampling, underlying medical condition, degree of immunosuppression, the species and serotype of infecting *Candida* and the type of serological test affects the detection of mannan in body fluids of DC patients (Ellepola and Morrison, 2005).

1, 3,-β-D-glucan (BDG) a polysaccharide, is a structural component of *Candida* cell wall (Odabasi *et al*., 2004). Its presence in the circulation of patients signifies systemic mycoses (Ellepola and Morrison, 2005). BDG can be detected by its ability to activate factor G of Japanese horseshoe crab (*Tachypleus tridentalis*) coagulation cascade (Obayashi *et al*., 1992). Commercially available Fungitec-G test (Seikagaku Corporation, Tokyo, Japan)

colorimetrically measures plasma BDG. In this test prior treatment of patient's plasma with perchloric acid is necessary to precipitate interfering factors (Obayashi *et al*., 1992). Although this test is incapable of identification of causative fungus, its rapidity makes it an attractive screening test for diagnosis of DC. The test may show false positive results in several conditions like haemodialysis, abdominal surgery and treatment with β-lactam antibiotics (Ahmad and Khan, 2012). The other antigens that can be detected in the DC patients are 47 kDA protein, enolase, specific mannosides and extracellular secreted proteinase (Sap).

Molecular diagnostic techniques

The time required for culture and speciation of *Candida* isolates necessitate the used of molecular techniques for diagnosis of DC. The conventional methods of culture and identification of *Candida* requires a minimum of one week. On the other hand, molecular diagnostic techniques are rapid, sensitive and specific (Sullivan *et al*., 1996).

Among various molecular procedures applied for identification of *Candida* spp., PCR technology appears to be precise and sensitive (Sullivan *et al*., 1996). PCR tests can rapidly detect most pathogenic *Candida* spp. by employing primers to DNA sequences that are common but unique to these organisms as a group. This technique can be applied for identification of *C. albicans* and commonly encountered NAC spp. (*C. tropicalis*, *C. glabrata*, *C. krusei* and *C. parapsilosis*) (Lehmann *et a*l., 1992; Gil-Lamaignere *et al*., 2003). PCR can detect extremely small quantities of fungal DNA with great accuracy and thus can be used for diagnosis DC during the early developmental stages of infection (Sullivan *et al*., 1996). Early diagnosis of DC is the key for initiation of appropriate treatment and prevention of morbidity and mortality. In contrast to other molecular techniques, PCR is the only technique that can be used for detecting *Candida* spp directly from clinical specimens (Sullivan *et al*., 1996).

In PCR technique false negative results are associated with samples such as thick pus, where DNA extraction may be difficult (Sullivan *et al*., 1996). False positive results may be due contamination of specimens with normal flora of patients. Hence PCR technology can be used only for detection of *Candida* spp. from normally sterile sites such as blood, CSF and peritoneal fluid.

The incidence of *Candida* infections has increased over the past few decades. It is now widely accepted that *Candida* species are a common cause of health care associated infection with significant associated morbidity and mortality. *Candida albicans* is the most common species implicated in infections, but in recent years non-*albicans* species are increasingly reported in various published literatures. For improved therapeutic outcome, the rapid and accurate diagnosis of etiological agent is very essential. Various conventional and advance laboratory methods are available for diagnosis of candidiasis. Advanced immunological and molecular techniques are significant for prompt and precise diagnosis of candidiasis; further evaluation of these techniques in different health-care set-ups is warranted.

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