World J Microbiol Biotechnol (2014) 30:1437–1451
DOI 10.1007/s11274-013-1574-z

REVIEW

Candida identification: a journey from conventional to molecular methods in medical mycology

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Received: 11 July 2013 / Accepted: 2 December 2013 / Published online: 1 January 2014

Abstract The incidence of *Candida* infections have increased substantially in recent years due to aggressive use of immunosuppressants among patients. Use of broad-spectrum antibiotics and intravascular catheters in the intensive care unit have also attributed with high risks of candidiasis among immunocompromised patients. Among *Candida* species, *C. albicans* accounts for the majority of superficial and systemic infections, usually associated with high morbidity and mortality often caused due to increase in antimicrobial resistance and restricted number of antifungal drugs. Therefore, early detection of candidemia and correct identification of *Candida* species are indispensable pre-requisites for appropriate therapeutic intervention. Since blood culture based methods lack sensitivity, and species-specific identification by conventional method is time-consuming and often leads to misdiagnosis within closely related species, hence, molecular methods may provide alternative for accurate and rapid identification of *Candida* species. Although, several molecular approaches have been developed for accurate identification of *Candida* species but the internal transcribed spacer 1 and 2 (ITS1 and ITS2) regions of the rRNA gene are being used extensively in a variety of formats. Of note, ITS sequencing and PCR–RFLP analysis of ITS region seems to be promising as a rapid, easy, and cost-effective method for identification of *Candida* species. Here, we review a number of existing techniques ranging from conventional to molecular approaches currently in use for the identification of *Candida* species. Further, advantages and limitations of these methods are also discussed with respect to their discriminatory power, reproducibility, and ease of performance.

Keywords Candida · Germ tube test · CMA · Candidaemia · RAPD · MALDI-TOF · RFLP · PNA FISH

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Introduction

Candida species are ubiquitous fungi and most common fungal pathogens infecting humans. Pathogenic *Candida* species were earlier confined to the human and animal reservoirs but they are now frequently recovered from the hospital environment, such as from foods, counter tops, air-conditioning vents, floors, respirators, and medical personnel (Ferreira et al. 2013; Sabino et al. 2011). They have predominantly unicellular mode of development. More than 200 species of *Candida* are known, but only a small proportion of them are found in man, and of these only a handful create clinical problems (Pappas 2006). An interesting feature of *C. albicans* is its ability to grow in two different ways: reproduction by budding, forming an ellipsoid bud, and in hyphal form, which can periodically fragment and give rise to new mycelia, or yeast-like forms (Fig. 1). It lacks a sexual cycle and is a diploid organism, which has made it difficult to manipulate genetically (Odds

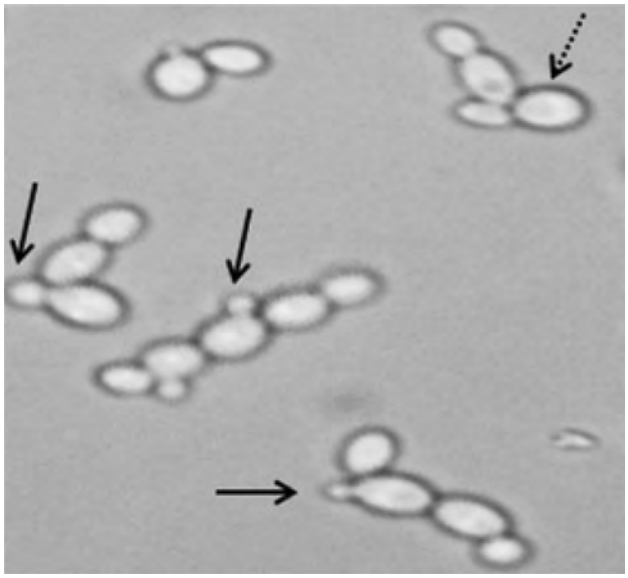

Fig. 1 *Candida albicans* showing budding (*solid arrow*) and yeast-like form (*dotted arrow*)

1988). The most critical criterion for pathogenicity of *C. albicans* is the induction of the mycelial form by serum or macrophages. Its pathogenicity has been attributed to its ability to switch between the yeast and hyphal mode of growth (Cutler 1991; Leberer et al. 1997; Lo et al. 1997). It is the most common fungal pathogen that exists as a harmless commensal in the gastrointestinal and genitourinary tracts in about 70 % of humans (Ruhnke and Maschmeyer 2002; Meiller et al. 2009; Schulze and Sonnenborn 2009; Sobel 1997). However, it becomes opportunistic pathogen in immunocompromised patients, or sub-immunocompromised individuals, or even some times in healthy persons (Alby et al. 2009; Pfaller and Diekema 2004). Among *Candida* species *C. albicans* accounts for the majority of systemic and superficial infections. The infection caused by *C. albicans* is commonly known as candidiasis and it can be classified into two categories depending upon the severity of the disease. The first category is characterized by mucosal infections, which generally affect gastrointestinal epithelial cells, oropharyngeal mucosa and vagina. Vulvo Vaginal Candidiasis (VVC) is common among women and some of them experiences repeated occurrences of this infection, which is known as Recurrent Vulvo Vaginal Candidiasis (RVVC). About 75 % of women suffer from *Candida* infection at least once in their lifetime. It can also cause life-threatening systemic infections to severely ill patients in whom mortality rate is about 30 % (Odds 1988; Soll et al. 1988; Calderone 2002; Sexton et al. 2007). Systemic *Candida* infections are common to immunocompromised individuals, including HIV-infected patients, transplant and chemotherapy recipients, and low-birth weight infants (Pfaller and Diekema 2007; Schelenz 2008). Although *C. albicans* is a major infectious agent responsible for candidiasis but some non-albicans species like *C. glabrata*, *C. krusei*, *C. dubliniensis*, *C. parapsilosis*, and *C. tropicalis* have also gain importance due to their frequent recovery from infected individuals (Gozalbo et al. 2004).

With the development in healthcare system worldwide, the number of elderly people and immunocompromised patients has increased dramatically. As a result, the rate of opportunistic infections caused by various microbes has also increased many folds. The growing interest of the scientific and medical communities in *Candida* sp. is reflected directly by a steady rise in the number of reports on the incidence of *Candida* infections over the past decade. It has been observed that *Candida* species are one of the four most common causes of bloodstream and cardiovascular infections in US hospitals (Gudlaugsson et al. 2003; Calderone 2002). Bloodstream infections caused by *Candida* are responsible for as high as 50 % mortality rate among the infected patients (Gudlaugsson et al. 2003; Eggimann et al. 2003b). In case of neonatal care units, *Candida* related bloodstream infections are even more frequent (Patel and Saiman 2010). Because of these outlined ailments, *C. albicans* has gained importance as a potential human pathogen, which warrants detailed study of this organism to understand its biology.

Epidemiology

Candida is the fourth pathogen, and the leading fungal pathogen responsible for bloodstream infections (BSI) in the USA and it accounts for 8–10 % of all BSIs acquired in the hospital (Wisplinghoff et al. 2004). In Europe, *Candida* is one of the major causes of nosocomial bloodstream infections (Marchetti et al. 2004; Fluit et al. 2000). According to a survey conducted between 1991 and 2000 by the Fungal Infection Network of Switzerland, ICUs and surgical wards accounted for about two-third of all episodes of candidaemia (Marchetti et al. 2004). According to the study of Falagas and his colleagues, the different surveillances shows that *C. albicans* was the predominant species in almost all studies. *C. albicans* predominated in the countries of North Europe and Switzerland (>60 %), was equally isolated in the remaining European countries and the USA ranging from 45 to 58 %, and was lower in Asia and South America approximately 40–42 %. On the other hand, non albicans species dominated in Asia. The highest proportions of *C. glabrata* were reported in studies from the USA (18.8–24 %) and the UK (22.7 %) and the lowest were reported in studies from Brazil and Kuwait 4.9 and 5.6 %, respectively. *C. parapsilosis* was more

frequently isolated in Kuwait (30.6 %), South America (20.5–21.3 %), Spain (23 %), Australia (19.9 %), and the lowest proportion was found in Switzerland (1–5 %) and North Europe (4.4 %). *C. tropicalis* held higher proportions in South America (20.9–24.2 %) and Taiwan (22.4 %), intermediate in the USA (11–12 %), and lowest in Central and North Europe (4 %) (Falagas et al. 2010).

The specific epidemiology regarding the incidence of candidaemia and distribution of *Candida* spp. (*C. albicans* versus non-albicans *Candida* spp.) varies markedly between institutions and patient cohorts (Luzzati et al. 2000; Slavin et al. 2004; Laupland et al. 2005; Lipsett 2006; Horvath et al. 2007). The Australian population-based surveillance report indicated *C. albicans* as the predominant species (47.3 %), with the next most frequent isolates being *C. parapsilosis* (19.9 %) and *C. glabrata* (15.4 %) (Chen et al. 2006). A study in a French burns unit identified *C. albicans* (65 %), *C. parapsilosis* (25 %), and *C. tropicalis* (10 %) as the common isolates in episodes of candidaemia. The attributable mortality rate for candidaemia in burns is reported as ranging from 14 to 70 % (Vinsonneau et al. 2009). In a retrospective study conducted in a university hospital in Saudi Arabia during the years 1991–2000 showed a total of 189 episodes of candidaemia, of which 121 (64 %) occurred during 1991–1995, whereas only 68 cases (36 %) were found between 1996 and 2000. *Candida albicans* was found to be the causal organism in 50.3 % episodes followed by *C. tropicalis* (27 %), *C. parapsilosis* (7.9 %), *C. glabrata* (7.4 %), *C. krusei* (3.2 %), *C. famata* (1.0 %). The percentage of episodes of candidaemia caused by *C. albicans* ranged from 36.4 % in 1991 to 71.4 % in 2000, revealing more than 100 % increase during the study period. The incidence of non *C. albicans* candidaemia decreased from 63 (33.3 %) during the first 5 years (1991–1995) to 31 (16.4 %) episodes during the second 5 years (Al-Hedaithy 2003). Interestingly, no candidaemia was occurred due to *C. glabrata* and *C. krusei* during the last 3 years. Overall, during the years of the study, a decreasing trend of candidaemia was observed. When analyzed with the underlying medical condition, candidaemia occurred more frequently in patients with leukemia (24 %), prematurity (16 %), post-surgery (10.6 %), and lymphoma (9.5 %). *Candida albicans* occurred more frequently (70–77 %) in patients with respiratory infections, preterm infants, TB, diabetes, and postoperative patients. On the other hand, non *C. albicans* species were more common among patients with leukemia (62 %), lymphoma (66.7 %), hepatic (77.8 %), and renal (62.5 %) disorders. Among patients with leukemia, *C. tropicalis* was isolated more frequently (42.2 %) followed by *C. albicans* (38 %). *Candida albicans* and *C. tropicalis* were the main species recovered from candidaemia in patients with respiratory infections. In the study, the involvement of *C. dubliniensis* for candidaemia was reported for the first time in Saudi Arabia. The age of patients in the study was ranged between 15 days to 80 years. The annual incidence of episodes of candidaemia during the years 1991–1995 were similar while that during 1996–2000 was decreased. A similar finding was also reported by another investigator from Riyadh, Saudi Arabia in which a total of 98 distinct episodes of candidaemia were identified during January 1996 to December 2004. The most frequent *Candida* species were *C. albicans* (53 %). Among non *C. albicans*, *C. tropicalis* was found to be involved in 19 % followed by *C. parapsilosis* (16 %) and *C. glabrata* (7 %). The overall crude mortality rate from the study was estimated at 43 % for all candidaemia (Al-Tawfiq 2007). In a study conducted at King Abdulaziz University hospital, Jeddah, Saudi Arabia, thirty-one candidemic episodes were identified. All the candidemic episodes were hospital acquired. The most common risk factors to candidaemia were central venous catheters (87 %), stay in intensive care unit (ICU) (77 %), and broad-spectrum antibiotics therapy (74 %). *Candida albicans* was the most frequently isolated species (71 %), followed by *Candida tropicalis* and *Candida parapsilosis* 13 % each (Akbar and Tahawi 2001). Although estimated attributable mortality rate of candidaemia from various studies around the globe is, more than 30 % (range 24–60 %, median 38 %). However, among different patient cohorts, the crude mortality rate has been reported to be more than 50 % (range 13–90 %, median 55 %) (Lemmen et al. 2000; Cochran et al. 2002; Eggimann et al. 2003a; Charles et al. 2005; Chen et al. 2006; Ballard et al. 2008).

Identification of *Candida*

Non-molecular methods

Conventional

Classical methods for the identification of yeast begin with the isolation of the microorganisms from the clinical specimens. Complete identification by conventional methods can take 24–48 h or even longer from blood culture. It is usually possible to distinguish *Candida* isolates from the culture medium using methods, which include the germ tube test, chlamydospore formation, and the fermentation or assimilation of sugars (Fig. 2). The germ-tube test is a rapid identification method for *C. albicans* (2–4 h), but it is not always 100 % percent accurate since approximately 5 % of *C. albicans* isolates do not produce germ tubes (Perry and Miller 1987; Odds 1988), while some *Candida tropicalis* isolates are also germ-tube producers (Martin

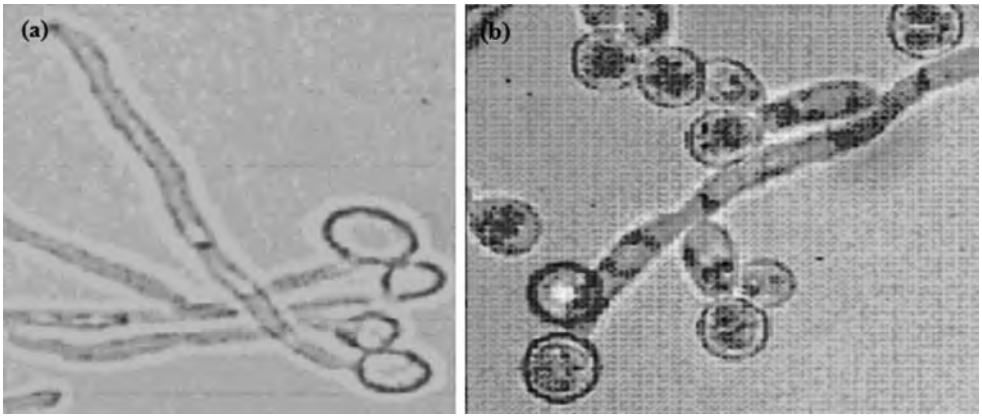

Fig. 2 *Candida albicans* showing **a** Germ tube formation in horse serum **b** Thick walled chlamydospores on CMA

and White 1981). The differentiation between *C. dubliniensis* and *C. albicans* is done on the basis of ability to grow on the Sabouraud dextrose agar (SDA) or broth medium containing 6.5 % NaCl. *C. albicans* grows well whereas *C. dubliniensis* are unable to grow at 6.5 % NaCl in the medium (Alves et al. 2002). Another method of differentiation between *C. dubliniensis* and *C. albicans* is the ability of *C. albicans* to grow at elevated temperatures. *C. dubliniensis* can grow well at 30 °C and 37 °C, producing creamy white colonies on solid media similar to *C. albicans*. However, differing from *C. albicans*, it grows poorly or unable to grow at 42 °C on SDA or potato dextrose agar (Sullivan et al. 1995). Moreover, this technique is dependent on the expertise of the technical personnel who perform the test and also affected by laboratory conditions (Fenn et al. 1996; Salkin et al. 1987). These factors complicate the accurate interpretation of this test. In the case of methods where the assimilation or fermentation of sugars is evaluated, complete identification can take 18–72 h. Conventional diagnostic procedures, such as blood culture and biochemical tests lack the degree of sensitivity and specificity that would ensure reliable and early diagnosis of invasive *Candida* infections (White et al. 2003; Stevens 2002). The importance of identifying the pathogens as rapidly as possible has lead to the development of differential media for the presumptive identification of yeasts in 1970s. Several chromogenic media for isolation and identification of *Candida* species has been developed and are available (Letscher-Bru et al. 2002). The basis of these media are the formation of different colored colonies with varied morphology which result from the cleavage of chromogenic substrates by species specific enzymes (Bauters and Nelis 2002). Examples of commercially available chromogenic agars include CHROMagar *Candida*, Fluroplate, Candichrom (Nickersok 1953), Pagano-Levin agar (Pagano et al. 1957), Costa-de Lourdes Branco (Costa and Brancocde 1964), and Albicans ID (Lipperheide et al. 1993), CHROMagar, BiGGY agar, Corn Meal agar etc. The growth morphology of different *Candida* species on Tween 80 Corn Meal agar is presented in Fig. 3. Commercial tests and kits available for *Candida* identification are summarized in Table 1.

Molecular methods

Non-DNA based techniques

Serological Serological methods for the diagnosis of invasive candidiasis were began some 60 years back when blood culture detection techniques often failed to recover *Candida* organisms (Bodey 1966; Myerowitz et al. 1977) and only antibody detection could provide useful data for the clinician (Taschdjian et al. 1973). The use of immunological tests with specific antisera has been described using either polyclonal or monoclonal antibodies (Brawner and Cutler 1984; Cassone et al. 1988; Guinet and Bruneau 1991; Hopwood et al. 1986; Polonelli and Morace 1986; Shinoda et al. 1981; Taguchi et al. 1979). A large number of assays has been developed and are commercially available for the detection of circulating *Candida* antigens such as latex agglutination, ELISA, immunoblotting, dot immunoassay, liposomal immunoassay, and RIA (Ponton et al. 2002). These assays depend on the detection of antigens, which include mannan and mannoproteins, glucan, HSP90, enolase, and other immunodominant cytoplasmic antigens. However, serological method has some limitations such as diagnosis is often delayed and the test lacks sensitivity and specificity. Furthermore, antibody detection for the diagnosis of candidiasis has been limited by false negative results in immunocompromised patients where there is low or undetectable level of antibody. This is due to the fact that fungal antigens and metabolites are usually cleared rapidly from the circulation. Moreover, the presence of antibodies does not always mean a *Candida*

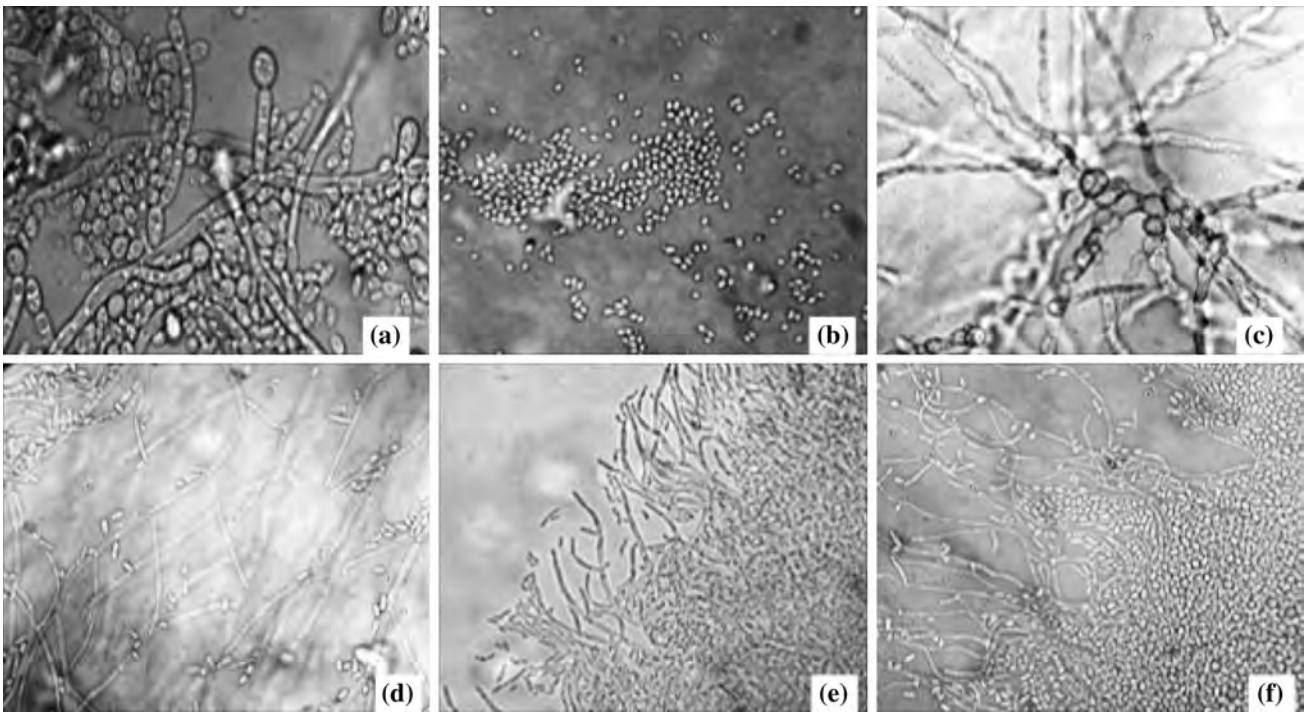

Fig. 3 The growth morphology of different *Candida* species on Tween 80 Corn Meal agar (CMA). **a** *C. albicans,* **b** *C. glabrata,* **c** *C. Parapsilosis,* **d** *C. krusei,* **e** *C. Kefyr* and **f** *C. tropicalis.* Cells were viewed under Zeiss microscope (Axioskop2 MOT model)

infection, particularly in patients with serious underlying disease or who are taking immunosuppressive drugs (Yeo and Wong 2002). The superficial colonization of *Candida* in some patients is the major reason of false positive results and thus consequently limits its wide use (Wahyuningsih et al. 2000a).

Spectroscopic There are several spectroscopic methods to identify the *Candida* such as Mass spectroscopy, FTIR, MALDI-TOF–MS.

(a) *Matrix-assisted laser desorption/Ionization-time of flight-mass spectrometry*

Mass spectrometry (MS), is a technique that separates molecules based on differences in mass/charge ratio. It was first described in 1912 by J. J. Thompson (Budzikiewicz and Grigsby 2006). This technique soon becomes a vital tool in the identification, quantization, and detection of small chemical structures. The mass spectrometry was first used to identify microorganisms in 1975 (Anhalt and Fenselau 1975). However, mass spectrometry technique got a setback from irreproducible results due to the variabilities caused by the growth conditions and media. The analysis of large biomolecules including ribosomal proteins became possible only in 1980s when matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) technique was coupled with mass spectrometry (Hillenkamp and Karas 1990). The later are less influenced by culture conditions allowing MALDI–TOF–MS to be consistently used to differentiate bacterial species (Claydon et al. 1996; Holland et al. 1996; Demirev et al. 1999; Fenselau and Demirev 2001; Krishnamurthy et al. 1996). Among the spectroscopic techniques, MALDI–TOF MS is the most widely used method to date for the analysis of biomolecules. MALDI–TOF–MS embeds the large biomolecules of interest in a crystalline matrix, usually a UV-absorbing organic acid (Lewis et al. 2000). When energy is added in the form of laser pulses with a wavelength matched to the absorption maximum of this matrix, this energy is efficiently transferred from the matrix to the analyte creating "charged vapor" of the biomolecules of interest (Adam 2002; Karas and Hillenkamp 1988). The particles in this vapor are accelerated and separated under the influence of electric field, requiring different times to reach the detector referred to as the time of flight. This creates a pattern of peak at the detector, the mass spectrum. Microorganisms are basically the complex mixtures of biomolecules and hence can be suitably subjected for MALDI–TOF–MS. The co-crystallization of a bacterial colony with the matrix is the only handling required before the analysis of sample with laser pulses. The resulting spectra are species-specific and highly reproducible as the analytical range 2–20 kDa mainly represents ribosomal proteins, which are always expressed and highly abundant (Ryzhov and Fenselau 2001). The spectrum is compared to a library of reference spectra, leading to identification. In

Table 1 Tests and kits available for identification of *Candida* species

Test/kit	Principle	Duration (h)
a. Conventional methods		
Germ tube test	Presence of hypha in *C. albicans*	24–36
Chlamydospore formation	Sporulation	24–72
Biochemical tests	Assimilation/fermentation of carbohydrates	48
API Candida	Assimilation/fermentation of carbohydrates	18/24
ID YST/VITEK 2	Biochemical tests	15
API 20C AUX	Assimilation tests	
Pagano-Levin Agar	Color change/reduction of triphenyltetrazolium to triphenylformazin	48
Candida ID Agar	Hydrolysis indolyl glucosaminide by *C. albicans*	48
Albican ID	A chromogenic substrate hydrolyzed by the hexosaminidase of *C. albicans*	>24
Fluoroplate	A fluorogenic substrate hydrolyzed by the hexosaminidase of *C. albicans*	>24
CHROM Agar	β-glucosaminidase metabolized to produce colored colonies of different *Candida* spp.	48
BiGGY Agar	Reduction of bismuth sulfite to bismuth sulfide	48
Corn Meal Agar	Stimulates sporulation in *Candida*	>48
Candida Diagnostic Agar	Glucosaminidase hydrolysis by different species of Candida to produce varying color	48
AlbiQuick	Detection of the enzymes β -galactosaminidase and L-proline aminopeptidase	>24
ChromID Candida	Hydrolysis of a hexosaminidase chromogenic substrate producing different colours	24
Candi*Select* 4	*Candida* identification based on specific enzymatic activity resulting in the formation of colored colonies	48
b. Molecular methods		
Serological	Immunological assay based antigen–antibody reaction against mannan, mannoprotien, glucan, HSP90, enolase etc.	>24
FTIR	Spectrum difference of protein among *Candida* species	>24
MALDI-TOF–MS	Spectrum difference of protein among *Candida* species	24
PNA FISH	Synthetic DNA mimics to hybridize with complementary DNA targets	>24
RFLP	Restriction digestion and hybridization with probe	48–72
Microsattelite typing	Based on PCR amplification	24
Multilocus typing	Based on PCR amplification	24
RAPD	Based on PCR amplification	<12

recent years, MALDI-TOF–MS has been implemented in routine laboratories and utilized as a completely new approach for the identification of bacteria and yeast. MALDI-TOF MS undeniably changed the laboratory practices in bacteriology. Since it is a very sensitive technique, only a small amount of microbial biomass is required for analysis. Several investigators have successfully identified *Candida* species using MALDI-TOF-MS. Veen and his colleagues evaluated the accuracy of MALDI-TOF-MS in identifying yeast isolates, including *Candida* (*C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. kefyr*, *C. krusei*, *C. parapsilosis*, *C. tropicalis*, *C. lusitaniae*), *Saccharomyces cerevisiae*, *Galactomyces geotrichum*, *Geotrichum* spp. *Magnusiomyces capitatus*, *Rhodotorula glutinis*, and *Trichosporon mucoides*. They reported that a total of 78 out of 80 isolates (97.5 %) were correctly identified at the genus level and 70(87.5 %) were identified at the species level. The remaining *Candida* species were not identified due to non availability of reference MALDI-TOF-MS spectra of the corresponding *Candida* species in the database (Veen et al. 2010). In another study, 247 out of 267 clinical *Candida* isolates from 15 different species were identified correctly by MALDI-TOF-MS (Marklein et al. 2009). In an investigation, a total of 103 isolates from *Candida parapsilosis*, *C. orthopsilosis* and *C. metapsilosis* were identified by pyrosequencing of the ITS1 region and then assayed by MALDI-TOF mass spectrometry. Concordance between these two methods was found to be 100 %, suggesting that MALDI-TOF may be useful as a rapid and reliable method for discrimination of species within the *C. parapsilosis* group (Quiles-Melero et al. 2012). Herendael and co-

workers reported correct identification of 163/167 (97.6 %) isolates by MALDI-TOF-MS (Herendael et al. 2012).

There are certain limitations for every diagnostic technique and MALDI-TOF-MS is no exception. The major limitation with MALDI-TOF-MS is the requirement of a pre-culture for successful analysis of patient samples. Another limitation includes inconsistencies between biochemical, molecular, and MALDI-TOF-MS based differentiation results. Since, in MALDI-TOF-MS technique, pathogen identification is based on the analysis of ribosomal protein spectra, species that do not differ adequately in their ribosomal protein sequences gives inconclusive or false identification. One such example, though not from *Candida*, is *Shigella* spp. and *Escherichia coli* or *Streptococcus pneumoniae* (*pneumococcus*) and members of the *Streptococcus oralis/mitis* group, which cannot be distinguished by MALDI-TOF-MS. Due to these shortcomings in this technique, other alternative method such as classical biochemical tests, antigen detection, or DNA based molecular methods are required. Further, the MALDI-TOF system has the inability to detect pathogens directly from patient material with the exception of urine. Various attempts are made to directly analyzed patient material from other sources, such as cerebrospinal fluid and blood. However, no working protocols have been published to date.

(b) *Fourier transform infrared spectroscopy.*

Fourier transform infrared spectroscopy (FTIR) characterizes the chemical composition of very complex biological systems such as microorganisms and provides highly specific spectroscopic fingerprints. This technique represents an analytical, non-destructive and dynamic method to investigate a population of whole cells with only little biomass (Naumann et al. 1991; Sandt et al. 2003). It permits identification and characterization of microbial cells at both species and sub-species level. The FTIR technique is sensitive that requires very little sample: ng–μg, relatively fast and simple to use. Little or no sample preparation is required for spectral acquisition. The *Candida* cells remains intact during analysis. The FTIR spectra provide information about cell composition and quantify the number or amount of functional groups present in a sample. Samples can be tested in the form of liquid, gas, powder, or film. Interestingly, *Candida* samples can be discriminated on the basis of their physiological state such as live, dead, injured, and treated. There are few limitations with this technique such as complex samples like mixture of *Candida* species produce overlapping spectra, which may lead to misinterpretation of results. Hence, a purification step is required. Therefore, a complete library of spectra for each type of *Candida* is required to facilitate detection. Moreover, culture medium, growth time, and growth temperature may cause variations in spectra. Presence of water in a sample may influence the bands at certain specific wave-number (Mariey et al. 2001; Burgula et al. 2007). The FTIR spectroscopy has been used to investigate differences in structure and content of components of the cell wall such as b-glucans, mannoproteins, and lipids of different *Candida* species (Dominique Toubas 2007; Essendoubi et al. 2005) .

PNA FISH Peptide nucleic acid (PNA) probes, developed in the early 1990s (Egholm et al. 1993; Nielsen et al. 1994) are synthetic DNA mimics where negatively charged sugar-phosphate backbone of DNA is replaced by an achiral, neutral polyamide backbone formed by repetitive units of *N*-(2-aminoethyl) glycine. Individual nucleotide bases are attached to each of the units providing a molecular design that enables PNA to hybridize to complementary nucleic acid targets according to the Watson and Crick base pairing rules. The synthetic backbone provides PNA probes with exceptional hybridization features, such as more rapid and stronger binding to complementary targets (Egholm et al. 1993). PNA is not degraded by nucleases or proteases (Demidov et al. 1994) and due to its relative hydrophobic character it penetrates the cell wall of yeast following fixation (Rigby et al. 2002; Stender et al. 2001). These chemical characteristics of PNA have been explored in a variety of research and diagnostic applications, such as point mutation analysis (Igloi 1999), chromosome analysis (Taneja et al. 2001), virology (Just et al. 1998), mycology (Stender et al. 2001), and bacteriology (Thisted et al. 1999). PNA probes can be combined in standard fluorescent in situ hybridization (FISH) format making it a powerful diagnostic tool. In 2004, FDA has approved PNA FISH technique for the identification of *C. albicans* directly from blood culture bottles in vitro (Alexander and Pfaller 2006). The probes utilized by PNA FISH technique are superior to conventional nucleic acid probes in their better binding properties and stability. PNA-DNA hybridization is affected by base mismatches and PNA can maintain sequence discrimination up to the level of a single nucleotide mismatch. On the other hand, base mismatches are less effective for corresponding DNA–DNA hybridization. These characteristics give PNA FISH a much higher specificity, making it an ideal tool to differentiate *C. albicans* in vitro (Egholm et al. 1993; Ray and Nordén 2000). Since this new detection technique targets highly conserved species-specific sequences in the abundant 26S rRNA of living *C. albicans,* individual cells can be detected directly without the need for amplification (Shepard et al. 2008). The PNA FISH is performed on microscope slides of direct blood smears and analyzed by fluorescence microscopy. Forrest and co-workers observed that PNA FISH significantly reduced the median time

required for the identification of *C. albicans* to 9.5 h (range 3–17 h), compared to the standard culture median time of 44 h (range 36–92 h), while the median time for the final identification of *Candida* species other than *C. albicans* by culture was even longer (61 h; range 36–124 h). However, the major limitation of the PNA FISH test is that it has not yet been validated with specimens other than blood. Moreover, only *C. albicans* specific probe is currently available. Development of probes for other *Candida* species will lead to greater clinical effectiveness of PNA FISH technique (Forrest et al. 2006; Chen et al. 2011).

DNA based techniques

The most widely used techniques among DNA based methods are the restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD) analysis, Multi locus sequence typing and Microsatellite typing. Compared to blood cultures and phenotypic methods, DNA based techniques have been adopted by several microbiological laboratories for rapid and objective identification of *C. albicans*. The sensitivity of a DNA based assay is dependent on sample preparation, primer and DNA target selection, extraction of DNA, and amplification effectiveness (Bretagne and Costa 2005). The success of amplification method is heavily dependent on the selection of targeted nucleic acid. Target DNA can either be obtained from databases using species-specific information or can be arbitrarily selected from the fungal genome. Nevertheless, species-specific sequences always give rise to better outcomes than arbitrary sequences. Highly conserved sequences of 5.8S, 18S and 28S ribosomal RNA genes are appropriate to differentiate *Candida* species. The internal transcribed spacer regions (ITS) located between these genes are very promising for the molecular identification of *C. albicans*, since they contain areas of high conservation as well as areas of high variability. Among them, ITS1 and ITS2 are primarily used, and the combination of these two length polymorphisms may yield better results than either one alone (Chen et al. 2001).

Microsatellite typing

Simple Sequence Repeats (SSRs) or microsatellites are tandemly repeated motifs of 1–6 bases found in all prokaryotic and eukaryotic genomes. They are present in both coding and non coding regions and are usually characterized by a high degree of length polymorphism (Hartl and Clark 1997; van Belkum 1999; Zane et al. 2002). Though the evolutionary mechanism of microsatellite still remains uncertain, even then microsatellites have been considered efficient genetic markers because of their high variability. The high reproducibility and stability of microsatellite markers have been reported by various researchers in their epidemiological studies involving the opportunistic pathogen *C. albicans* (van Belkum 1999; Zane et al. 2002). However, only a few polymorphic microsatellite loci have been identified in the genome of *C. albicans* located mostly next to or within coding regions and exhibiting a high discriminatory power (Botterel et al. 2001; Bretagne et al. 1997; Garcia-Hermoso et al. 2007). The microsatellites found in non-coding regions exhibits greater degree of polymorphism than those found within coding regions. Unfortunately, there are only few studies for analysis of these regions in *C. albicans* (Lott and Effat 2001; Lunel et al. 1998; Sampaio et al. 2003). Several researchers have exploited this technique and its different variants such as Multi-locus microsatellite typing (MLMT) for typing different strains from the same species of *Candida* (Chávez-Galarza et al. 2010; Sampaio et al. 2003; Ge et al. 2012).

Multi locus sequence typing

Multi locus sequence typing (MLST) directly measures the DNA sequence variations in a set of housekeeping genes and characterizes strains by their unique allelic profiles. The technique involves PCR amplification followed by DNA sequencing. Nucleotide differences between strains can be checked at a variable number of genes depending on the degree of discrimination desired. This technique has been shown to be a highly discriminatory and reproducible method for unambiguous specific strain characterization of *C. albicans* (Bougnoux et al. 2002; Bougnoux et al. 2006; Odds 2010). This method has now been successfully applied to study epidemiology, population genetics, and molecular phylogeny for differentiation of strains of *Candida tropicalis* and *Candida albicans*. MLST data for *C. albicans* strains are available in a public database that provides an indispensable resource to evaluate the worldwide diversity of *C. albicans* and the relationships of isolates identified at various locations (Odds et al. 2007; Tavanti et al. 2005).

Randomly Amplified Polymorphic DNA

The Randomly Amplified Polymorphic DNA (RAPD) assay uses a single or a pair-wise combination of primers, typically 9–10 nucleotides in length, to amplify target genomic DNA by the PCR. Fragments of DNA are generated by PCR amplification if the target sites for the primer happen to occur within approximately 5 kb of each other on opposite DNA strands. Since arbitrary sequence primers are used, RAPD fingerprints are relatively easy and fast to obtain. Furthermore, no prior knowledge is required for the DNA to be analyzed. This approach has been used by several researchers to type and identify *Candida*

species. Melo 1998 observed RAPD results for identification of *C. albicans, C. tropicalis, C. parapsilosis, C. glabrata* and *C. krusei* were 100 % consistent with the results obtained by conventional diagnostic methods (Melo 1998). Melting curve of random amplified polymorphic DNA (McRAPD) has been used to identify *Candida lusitaniae* isolates (Hamal et al. 2011). Xu and his colleagues have successfully genotyped 30 *Candida albicans* isolates through RAPD method by employing OPE-03 primer (Xu et al. 2012). In a study, 38 clinical isolates selected which were previously identified as *C. parapsilosis* and were subjected to the RAPD analysis using CA2 primer. Following RAPD analysis, 4 were re-identified as *C. metapsilosis* and 5 as *C. orthopsilosis* (del Pilar Vercher et al. 2011). RAPD method using primers CA1 and CA2 exhibited the discriminatory power by discriminating 22 genotypes for *Candida albicans* with CA1 oligonucleotides and 19 genotypes with CA2 primer but when both primers CA1 and CA2 were combined, 17 genotypes were obtained for *C. glabrata* (Noumi et al. 2009). It is also suggested that RAPD may be employed to establish the relationship between genotype and drug susceptibility of *C. albicans* simultaneously (Jain et al. 2001; Xu et al. 2012).

Restriction fragment length polymorphism

Restriction fragment length polymorphism (RFLP) is a type of polymorphism that results from variation in the DNA sequence recognized by restriction enzymes. These are bacterial enzymes used to cut DNA molecules at known locations. RFLPs are used as markers on genetic maps. This technique has been used by several researchers to type and identify *Candida* species by targeting different genes or probes such as CARE2, 27A, Ca3 or internal transcribed spacers (ITS1 and ITS2), 5.8S rRNA gene, 26S D1/D2 domain, etc. with different sets of restriction enzymes. Ca3 is a moderately repetitive DNA fragment of *Candida albicans* genome. Digestion of Ca3 DNA probes generates a pattern of 15–25 bands of various intensities depending on the restriction enzyme used. Fingerprinting by Ca3 probe has proven effective in a number of epidemiological studies involving significant numbers of strains (Schmid et al. 1990, 1992, 1995). Another probe, CARE2 is 1.06 kb moderately repetitive fragment of DNA, which has been widely utilized for the identification of *Candida* species. Reef et al. (1998) analyzed 49 *C. albicans* isolates to determine the mode of acquisition of infection in hospitalized patients by restriction fragment length polymorphism analysis using genomic blots hybridized with the CARE-2 probe (Reef et al. 1998). But these methods suffers with certain limitations such as they are more expensive and time consuming as well as needs skilled workers trained in such techniques to interpret the data.

Nucleic acid analysis of Internal transcribed spacer (ITS) region of rRNA

Although, several molecular approaches have been developed to provide more rapid and accurate identification of *Candida* species as compared to traditional phenotypic methods, but the internal transcribed spacer 1 and 2 (ITS1 and ITS2) regions of the rRNA gene have gained upper hand for typing *Candida* species in a variety of approaches. These methods include PCR (Li et al. 2003; Luo and Mitchell 2002), ITS fragment length polymorphism (Chen et al. 2000, 2001; Turenne et al. 1999), restriction fragment length polymorphism (Frutos et al. 2004; Majoros et al. 2003; Trost et al. 2004), DNA probe hybridization and DNA sequencing (Coignard et al. 2004; Elie et al. 1998; Lindsley et al. 2001; Martin et al. 2000; Wahyuningsih et al. 2000b). Park and his colleagues has developed a molecular probe for rapid identification of *C. dubliniensis* as well as *C. albicans* by analyzing ITS2 region of rRNA genes from a reference *Candida* strains (Park et al. 2000). Frutos and co-workers has reported correct identification of *Candida* species by restriction analysis of the ribosomal region spanning ITS1 and ITS2 and the 5.8S rRNA gene (Frutos et al. 2004). Interestingly, one of the studies showed species differentiation among *Candida glabrata*, *C. nivariensis* and *C. bracarensis* on the basis of fragment length polymorphism of ITS1 and ITS2 (Mirhendi et al. 2011). Among several methods, ITS sequence analysis has been proven to be an accurate method for species identification because of its simplicity to perform due to being fully automated as well as nucleic acid interpretation is very straightforward and does not require very skilled expertise. The ITS1 and ITS2 regions of *Candida* species possesses enough nucleic acid sequence diversity which facilitates species-level identification of organisms by enabling targeted DNA sequencing of distinct regions within the rRNA gene complex (Iwen et al. 2002; Lott et al. 1998; Chen et al. 2000; Mercure et al. 1993). Since the ITS1 and ITS2 regions are highly informative and their flanking ribosomal genes are highly conserved, they are desirable targets for PCR–based amplification coupled with either DNA sequencing or species-specific hybridization probes (Einsele et al. 1997; Elie et al. 1998; Gharizadeh et al. 2004). Therefore, a practical approach for accurate and timely identification of *Candida* species includes DNA sequencing of the ITS1 or ITS2 regions. The TS1 and ITS2 have been used by several investigators for the purpose of fungal identification due to the sufficient extent of sequence variation in specific non-coding sequences (Hinrikson et al. 2005; Leaw et al. 2006; Schabereiter-Gurtner et al. 2007). In a study, Bayonton and coworkers were able to identify all 60 *Candida* isolates correctly at species-level through DNA pyrosequencing of ITS2 region

and sequencing interpretations were agreed in all cases with results of biochemical and morphologic testing. Different *Candida* species were identified, such as *C albicans*, *C dubliniensis*, *C glabrata*, *C guilliermondii*, *C krusei*, *C lusitaniae*, *C parapsilosis*, and *C tropicalis* (Boyanton et al. 2008). In another study, the ITS1 and ITS2 regions were sequenced from a large collection of 373 medically relevant yeast isolates (including *Candida* species), and the ITS2 region was the DNA target yielding 99.7 % correct species identification (Leaw et al. 2006). Although ITS sequencing holds a great promise for the correct identification of medically known important yeast species whose sequence database is available to compare with the subjected sequence in the medical diagnostic laboratory. But recently there are reports of the use of polymerase chain reaction–restricted fragment length polymorphism (PCR–RFLP) as a rapid, easy and cost-effective method for the identification of *Candida* species from blood isolates of ICU patients (Vijayakumar et al. 2012). In this method the ITS1-5.8S-ITS2 rDNA region from genomic DNA of *Candida* species were amplified by PCR. RFLP was performed for the amplified products using the *Msp* I restriction enzyme. Vijayakumar et al. (2012), have reported correct identification of the all isolates included in their study to the species level *such as C.tropicalis, C. albicans, C. parapsilosis, C. glabrata, C. kruse* from blood isolates of ICU patients (Vijayakumar et al. 2012). As a matter of fact, PCR–RFLP has been found to be a rapid and trustworthy method to speciate *Candida* isolates as evident by other studies. In a study by Shokohi and colleagues *Candida* species have been identified in cancer patients by PCR–RFLP using two restriction enzymes (*Bln* I, *Msp* I) (Shokohi et al. 2010). In another similar study, researchers have developed one-enzyme PCR–RFLP assay for identification of six medically important *Candida* species (Mirhendi et al. 2011). Interestingly, Okhravi and coworkers has shown PCR–RFLP analysis to speciate *Candida* isolates causing intraocular infections (Okhravi et al. 1998). Hence, this method seems to be very robust in terms of their rapidness, simplicity and cost-effectiveness and thus can be utilized in the routine laboratory diagnostics for the identification of *Candida* isolates which often posed significant challenges for the clinicians to prescribe appropriate antifungal therapy base on correct identification to manage the candidemia patients whose lives are always at a great risk.

Conclusion

Management of *Candida* infection remains severely hampered by delay in diagnosis. The conventional (classical) methods routinely used in hospital setup to identify *Candida* species are not much accurate as compared to molecular methods. Moreover, they are time consuming, usually take 2–5 days, and sometimes misdiagnose the species. In this article, we have focused comprehensibly on the techniques available so far in the literature regarding identification of *Candida* isolates. We have made an attempt to analyze the best possible and more trustworthy techniques and tools as an alternative choice for identification of clinical *Candida* isolates. Here, we focused to address a broad range of molecular techniques that have been used for identification and typing *Candida* species including DNA and non-DNA based methods. DNA-based methods generate unambiguous and highly reproducible typing data such as microsatellite length polymorphism and multi-locus sequence typing. In the review, molecular methods used for *Candida* species identification as well as strain typing are discussed in detail. The advantages and limitations of these methods are also discussed with regard to their discriminatory power, reproducibility, cost effectiveness, and ease of performance. Conventional identification of yeasts can be considered adequate for identifying species commonly encountered in clinical specimens, though it is time consuming and not very accurate especially with closely related homologous species and require expertise that is more technical. Molecular identification methods may be more expensive, but are time efficient and more accurate. Therefore, early and accurate diagnosis of *Candida* infection by DNA-fingerprinting method is indispensible for timely intervention with appropriate antifungal therapy. The polymerase chain reaction can be adopted for fingerprinting *Candida* by using random primers based on the analysis of *Candida* genome. RAPD analysis may also be a promising tool for *Candida* typing as well as for strain discrimination especially to evaluate the nosocomial infection. This method has the advantage that no prior sequence information is required, and particularly promising because of their simplicity, specificity, sensitivity, and rapid screening. In coming years, RAPD may also be proved promising tool in determining the relationship between genotype and drug susceptibility of *Candida species* simultaneously. Unfortunately, there is lack of research work focused on establishing the RAPD tools for simultaneous discrimination of resistant isolates from the sensitive ones along with species identification. There have been extensive reports of utilizing the internal transcribed spacer 1 and 2 (ITS1 and ITS2) regions of the rRNA gene for detection of *Candida* species in a variety of method. Among these molecular methods, the feasibility of using ITS sequencing for identification of medically important yeasts has been extensively carried out and has been proven to be an accurate method for species identification. However, ITS2 sequence seems to be more species specific than the ITS1 sequence, and almost all clinically

relevant species could be identified by using the ITS2 region alone. Recently, there are numerous reports of the use of polymerase chain reaction–restricted fragment length polymorphism (PCR–RFLP) of ITS rDNA as a rapid, easy, and cost-effective method for the rapid identification of *Candida* species.

Acknowledgments Authors are grateful to Mr. Mohammad S. Gazdar, Librarian, KFMRC, for providing assistance in retrieving research articles from journals available in the library and as well from different web resources.

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