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## ORIGINAL ARTICLE

# Phenotypic methods and commercial systems for the discrimination between *C. albicans* and *C. dubliniensis*

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***Candida dubliniensis* is a recently described *Candida* species associated with oral candidosis that exhibits a high degree of phenotypic similarity to *Candida albicans*. However, these species show differences in levels of resistance to antimycotic agents and ability to cause infections. Therefore, accurate clinical identification of *C. dubliniensis* and *C. albicans* species is important in order to treat oral candidal infections. Phenotypic identification methods are easy-to-use procedures for routine discrimination of oral isolates in the clinical microbiology laboratory. However, *C. dubliniensis* may be so far underreported in clinical samples because most currently used identification methods fail to recognize this yeast. Phenotypic methods depend on growth temperature, carbon source assimilation, chlamydospore and hyphal growth production, positive or negative growth on special media and intracellular enzyme production, among others. In this review, some phenotypic methods are presented with a special emphasis on the discrimination of *C. dubliniensis* and *C. albicans*.**
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**Keywords:** candidiasis, oral; *Candida*; *Candida albicans*; pathology, oral; isolation and purification; *Candida dubliniensis*

## Introduction

*Candida dubliniensis* is a recently described species of the genus *Candida* (Sullivan *et al*, 1995) primarily associated with oral candidosis (Sullivan and Coleman, 1998) in human immunodeficiency virus (HIV)-infected and acquired immunodeficiency syndrome (AIDS) patients (Gugnani *et al*, 2003; Sullivan *et al*, 2004) and diabetic patients (Manfredi *et al*, 2002). *C. dubliniensis* has also been identified as the cause of systemic disease in patients after bone marrow transplant, immunosuppressive treatment or cytotoxic chemotherapy for the treatment of rhabdomyosarcoma (Meis *et al*, 1999). This species was identified as a germ-tube- and chlamydospore-positive yeast with high similarity to *C. albicans* and *C. stellatoidea*. *C. dubliniensis* presents a decreased capacity to form hyphae and increased levels of proteinase production when compared with *C. albicans* (Gilfillan *et al*, 1998). *C. dubliniensis* also differs from *C. albicans* in its assimilation profile of carbohydrates and its growth temperature range (Coleman *et al*, 1997). According to Gilfillan *et al* (1998), *C. dubliniensis* adheres to human buccal epithelial cells *in vitro* to a greater extent than *C. albicans* in the presence of glucose, although *C. albicans* isolates are less adherent than *C. dubliniensis* to Vero cells (Borg-von Zepelin *et al*, 2002). An intrinsic resistance of *C. dubliniensis* to fluconazole has also been demonstrated (Moran *et al*, 1997; Ruhnke *et al*, 2000; Martinez *et al*, 2002; Perea *et al*, 2002) as well as an increased resistance to clinically used amphotericin B (Ramage *et al*, 2001).

The frequency of severe infections among immunocompromised or hospitalized patients is encouraging the optimization of methods of identification to permit an earlier recognition of pathogens and the administration of effective antifungal therapy (Pfaller *et al*, 1999). Moreover, the widespread geographical distribution of *C. dubliniensis* calls for a prospective epidemiological survey of this species in the world (Sullivan *et al*, 1997). As *C. dubliniensis* continues to gain importance as an opportunistic pathogen and in light of its predisposition for fluconazole resistance, it has become important for laboratories to screen for this species in clinical specimens (Jabra-Rizk *et al*, 2000).

Molecular techniques are very reliable and precise for candidal discrimination (Soll, 2000), but relatively onerous and impractical for the great majority of the laboratories. Phenotypic identification methods are cost-effective and practical procedures for routine discrimination of oral isolates in the clinical microbiology laboratory (Al Mosaid *et al*, 2001; Alves *et al*, 2002). However, given the variability in the phenotypic

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characteristics and other irregularities in the taxonomy of the genus *Candida* (Coleman *et al*, 1997), identification based on the traditional methods has become less reliable. Nevertheless, important progress has been made with various studies on the phenotypic characteristics of these species and using commercially available identification systems. Moreover, wherever utilized in conjunction with each other these methods could be of value in the ultimate identification of *C. dubliniensis* and *C. albicans* (Williams and Lewis, 2000). The aim of this review is to list and discuss the main phenotypic methods and commercial systems currently available for the identification of *C. albicans* and *C. dubliniensis* from the oral cavity.

## Phenotypic methods for discrimination between *C. albicans* and *C. dubliniensis*

*Agar methods*

The screening methods to differentiate among *Candida* species include plating clinical samples onto solid media (Contreras *et al*, 1996). They contain a chromogenic or a fluorogenic substrate that is hydrolyzed by specific enzymes that allow macroscopic identification of *Candida* species on the basis of the color or fluorescence of the colonies (Contreras *et al*, 1996; Anson and Allen, 1997; Velegraki and Logotheti, 1998; Pincus *et al*, 1999; Willinger and Manafi, 1999; Willinger *et al*, 2001).

The use of the CHROMagar® Candida (CA) medium (CHROMagar, Paris, France) is advantageous to facilitate the detection of mixtures of yeast species from different samples on a single isolation plate (Anson and Allen, 1997). This medium is particularly useful as *C. dubliniensis* is often co-isolated with *C. albicans*. Moreover, CA proved to be cost-effective in laboratories with large numbers of isolates (Ainscough and Kibbler, 1998). CA allows selective yeast isolation identifying colonies of *C. albicans*, *C. tropicalis*, *C. krusei* and *C. glabrata* by morphology and color reaction (Odds and Bernaerts, 1994; Pfaller *et al*, 1996; Willinger and Manafi, 1999). *C. dubliniensis* develops dark green colonies after incubation at 37°C for 48 h, in contrast to the light green, light-blue or blue green colonies seen with *C. albicans* (Pincus *et al*, 1999; Williams and Lewis, 2000; Willinger *et al*, 2001) (Table 1). However, discrimination between these two species using CA appears to decline on a subculture and storage at −70°C of isolates (Pincus *et al*, 1999), with *C. dubliniensis* colonies exhibiting the light green appearance of *C. albicans*. This might be related to the ability of *C. dubliniensis* to exhibit the phenomenon of phenotypic switching (Sullivan and Coleman, 1998). Moreover, Tintelnot *et al* (2000) concluded that the sole use of green color on CA was insufficient for selective isolation of *C. dubliniensis*, since only 30 of 53 proven *C. dubliniensis* isolates showed a dark green color in primary cultures. Similarly, Borst *et al* (2003) found a 6% misidentification rate of *C. dubliniensis* as *C. albicans* when screening clinical isolates.

Candida ID (BioMérieux, Marcy l'Etoile, France) is a differential chromogenic medium for the direct identification of *C. albicans*, which produces blue-green colonies in contrast with the pink colonies produced by *C. tropicalis*, *C. guilliermondii* and *C. kefyr* (Willinger *et al*, 2001) (Table 1). According to Willinger *et al* (2001), after a 48-h incubation period, dark-bluish green colonies may be taken as an indication of the presence of *C. dubliniensis* but may not be used as a sole criterion for identification of this microorganism.

The use of a tetrazolium agar medium was suggested by Velegraki and Logotheti (1998) as a rapid, simple and inexpensive test for screening and presumptive identification of *C. dubliniensis*. This test differentiates between *C. dubliniensis* which produces red to maroon colonies and *C. albicans* which produces pale pink to whitish colonies when cultured onto tetrazolium salt embedded in a solid agar medium. The authors suggested that a concomitant positive germ tube test can produce evidence of the presence of *C. dubliniensis* in clinical samples.

**Table 1** Agar media used to presumptive discrimination between *C. albicans* and *C. dubliniensis* isolates

| *Agar* | *Basis of differentiation* | *C. albicans* | *C. dubliniensis* | *Reference* |
|---|---|---|---|---|
| Cornmeal–Tween 80 medium; rice agar–Tween medium; Pal's medium | Chlamydospore production | Small numbers, occurred singly and attached terminally to pseudohyphae | Large numbers and arrangement in contiguous pairs, triplets or larger multiples attached to a single suspensor cell | Sullivan *et al*, 1995; Sullivan and Coleman, 1998; Al Mosaid *et al*, 2001, 2003; Khan *et al*, 2004 |
| Casein medium; Staib medium | Chlamydospore production | Chlamydospore absent | Chlamydospore abundant | Staib and Morschhäuser, 1999; Al Mosaid *et al*, 2001; Lees and Barton, 2003; Mosca *et al*, 2003 |
| Tetrazolium salt médium | Colony color determined by the ability to reduce the tetrazolium salt | Pale pink to whitish colonies | Red to maroon colonies | Velegraki and Logotheti, 1998 |
| Chromagar Candida (Chromagar, Paris, France; M-Tech Diagnostics Ltd, Cheshire, UK) | Colony color determined by *β-N*-acetylgalactosaminidase activity | Light green, light-blue or blue green colonies | Dark green colonies | Coleman *et al*, 1997; Pincus *et al*, 1999; Williams and Lewis, 2000; Willinger *et al*, 2001 |
| Candida ID (BioMérieux, Marcy l'Etoile, France) | Colony color determined by hexosaminidase activity and other chromogenic substrate | Blue-green colonies | Dark-bluish green colonies | Willinger *et al*, 2001 |

*Carbohydrate assimilation tests*

The carbohydrate assimilation tests determine the ability of a yeast isolate to utilize a particular carbohydrate substrate as its sole carbon source in a medium. Growth of the yeast may occur in a liquid medium or in the vicinity of the carbon source on solid medium. Lack of growth indicates lack of enzymes for utilizing the test carbohydrate (Sandven, 1990). Several studies showed that the assimilation of glycerol, D-xylose, methyl-α-D-glucoside and D-trehalose could be used to distinguish both species by the inability of *C. albicans* to assimilate glycerol and the inability of *C. dubliniensis* to assimilate these three carbohydrates (Gales *et al*, 1999; Pincus *et al*, 1999; Ahmad *et al*, 2004). Although there is evidence that *C. dubliniensis* may sometimes be D-trehalose or to a minor extent methyl-α-D-glucoside positive (Ellepola *et al*, 2003). Unlike *C. albicans*, *C. dubliniensis* presents a lack of β-D-glucosidase activity, and this trait was suggested for the discrimination between these species (Tintelnot *et al*, 2000). Particularly, D-xylose may be helpful for the identification of *C. dubliniensis* (Gales *et al*, 1999; Ellepola *et al*, 2003; Fotedar and Al-Hedaithy, 2003).

Various commercially available yeast identification systems depending primarily on adaptations of carbohydrate assimilation procedures have been developed for *C. albicans* identification (Espinel-Ingroff *et al*, 1998; Gales *et al*, 1999; Pincus *et al*, 1999; Xu *et al*, 2002). The carbohydrate assimilation is examined either visually for changes in turbidity or color of pH indicators, or read automatically by using a photometer (Sandven, 1990). Table 2 shows the ability of *C. dubliniensis* to assimilate the various substrates compared with the substrate assimilation profiles of *C. albicans*.

The API 20C yeast identification system (BioMérieux) was considered as a reliable method for 19 assimilation tests for the identification of most of the clinically important yeasts after 72 h of incubation (Espinel-Ingroff *et al*, 1998). However, misidentification of *C. dubliniensis* as *C. albicans* (Xu *et al*, 2002) or 'no identification' of a *C. dubliniensis* isolate (Jabra-Rizk *et al*, 1999a) was found using this method. It was suggested that *C. dubliniensis* could be differentiated from *C. albicans* by its inability to assimilate xylose and methyl-α-D-glucoside in the API 20C system (Salkin *et al*, 1998). However, the API 20 C system is no longer commercially available and has recently been replaced by the API 20C AUX, which includes *C. dubliniensis*-specific carbohydrate assimilation profiles in the databases. Pincus *et al* (1999), found that the assimilation of glycerol, D-xylose, methyl-α-D-glucoside and D-trehalose in the API 20C AUX system could be used to distinguish *C. albicans* and *C. dubliniensis*. According to Sancak *et al* (2003) isolates showing no growth after 48 h at 42°C, *C. dubliniensis*-specific carbohydrate assimilation profiles with the API 20C AUX yeast identification system and chlamydospore production on cornmeal agar are highly likely to be *C. dubliniensis* rather than *C. albicans*.

Because of its high costs, the automated identification system Vitek Yeast Biochemical Card (YBC) (bioMeriéux SA) is more suitable to laboratories processing large numbers of specimens (Williams and Lewis, 2000). This system is able to differentiate between isolates of *C. albicans* and *C. dubliniensis* specifically by the inability of it to assimilate xylose and methyl-α-D-glucoside (Gales *et al*, 1999). Pincus *et al* (1999) found that while at 24 h, the profiles obtained with the Vitek YBC system showed that methyl-α-D-glucoside, D-xylose and glycerol could be used to separate the two species, at 48 h only D-xylose could be used for the same purpose. The Vitek 2 ID-YST is a fully automated system, with a database that comprises *C. dubliniensis*. The profit of correct identification is similar to that of other commercial systems and may be increased if morphological characteristics are evaluated (Graf *et al*, 2000). According to Gales *et al* (1999), clinical laboratories could use the absence of growth at 45°C and a negative D-xylose test with either the API 20C AUX or Vitek system to provide a presumptive identification of *C. dubliniensis*. A negative methyl-α-D-glucoside test result with both systems would also be helpful but may misclassify *C. albicans* as *C. dubliniensis*.

**Table 2** Phenotypic criteria for presumptive discrimination between *C. albicans* and *C. dubliniensis* isolates

| *Phenotypic criteria* | *C. albicans* | *C. dubliniensis* | *Reference* |
|---|---|---|---|
| Growth at 42 to 45°C | + | – | Coleman *et al*, 1997; Gales *et al*, 1999; Williams and Lewis, 2000; Sancak *et al*, 2003 |
| Growth on hypertonic Sabouraud broth | + | – | Alves *et al*, 2002; Ahmad *et al*, 2004; Sullivan *et al*, 2004 |
| Growth on glycerol | – | + | Pincus *et al*, 1999 |
| Growth on D-xylose | + | – | Gales *et al*, 1999; Pincus *et al*, 1999; Ellepola *et al*, 2003; Fotedar and Al-Hedaithy, 2003 |
| Growth on methyl-α-D-glucoside | + | – | Pincus *et al*, 1999; Ellepola *et al*, 2003 |
| Growth on D-trehalose | + | – | |
| β-D-glucosidase activity | + | – | |

+, positive growth or activity; –, negative growth or activity.

The ID 32C system (BioMérieux) records the ability of isolates to assimilate a variety of compounds that can be used as the sole source of carbon or nitrogen (Coleman *et al*, 1997). Results of Pincus *et al* (1999) with this system at 48 h showed that D-xylose, methyl-α-D-glucoside, lactate and D-trehalose could be used to separate *C. albicans* and *C. dubliniensis*. The ID 32 C system allows the evaluation of the assimilation of 29 carbon sources, growth in the presence of actidione (cycloheximide), and an esculin test. Numerical profiles are constructed from the reaction patterns and used to obtain identification with the identification software program. According to Freydiere *et al* (2001), the ID 32 C system correctly identified *C. dubliniensis*. Rapid ID 32 C consists of the automated ID 32 and rapid ID 32 identification test strips and includes the *C. dubliniensis*-specific carbohydrate assimilation profiles in the databases.

The RapID yeast Plus System (Innovative Diagnostic Systems, Norcross, GA, USA) is a qualitative micromethod that uses conventional and chromogenic substrates for fungal identification (Kitch *et al*, 1996). This method was suggested as an accurate, cost-effective and rapid (about 4 h) tool for the identification of common and emerging *Candida spp* (Espinel-Ingroff *et al*, 1998). The system database does not include data for *C. dubliniensis*, although Pincus *et al* (1999) had suggested that it is potentially able to separate this species from *C. albicans* by difference in reactivity on the assimilation of α,D-glucosidase and phosphatase. Results for α,D-glucosidase and phosphatase with *C. dubliniensis* strains were 23 and 9%, respectively, whereas the database values for *C. albicans* are 94 and 76%, respectively. However, the lack of data promoted several misidentifications of *C. dubliniensis* isolates.

*Germ-tube and chlamydospores production tests*

The germ tube test is a simple, efficient and economic test that is available to laboratory practitioners for screening and identification of *C. albicans*. Approximately 95–97% of *C. albicans* produce germ tubes when incubated in serum at 37°C for 2–4 h. However, in the same conditions, *C. dubliniensis*, *C. stellatoidea*, and *C. tropicalis* are also capable of inducing hyphal outgrowths. Similarly, *C. dubliniensis* and *C. albicans* are both capable of chlamydospores production (Meis *et al*, 1999), although the former frequently develops large numbers and arrangement of chlamydospores in contiguous pairs, triplets or larger multiples attached to a single suspensor cell (Sullivan *et al*, 1995; Coleman *et al*, 1997). In a study by Mosca *et al* (2003), 97.2% of *C. dubliniensis* isolates tested on casein agar medium at 24°C for 48 h produced abundant chlamydospores, whereas 92.5% of the *C. albicans* isolates tested failed to produce any chlamydospores (Table 1).

Al Mosaid *et al* (2003) and Khan *et al* (2004) suggested a sunflower (*Helianthus annus*) seed extract agar medium (Pal's Agar Medium), originally developed for the identification of *Cryptococcus neoformans*, for the discrimination of *C. albicans* and *C. dubliniensis* (Table 1). On this medium, *C. dubliniensis* but not *C. albicans*, presents a hyphal fringe surrounding colonies 24 to 72 h of incubation.

Niger Seed agar (Staib agar) has also been used as a medium for the differentiation of *C. albicans* and *C. dubliniensis* (Table 1). On this medium, *C. dubliniensis* forms rough colonies due to hyphal growth and produces abundant chlamydospores whereas *C. albicans* grows in smooth colonies and without chlamydospore formation (Staib and Morschhauser, 1999). Al Mosaid *et al* (2001) found 97.7% of *C. dubliniensis* strains growing as rough colonies and no strains of *C. albicans*. *C. albicans* produces only yeast cells on Staib agar after 24 h at 37°C, while *C. dubliniensis* produces extensive hyphal and pseudohyphal growth that is easily observed (Lees and Barton, 2003) (Figures 1–3).

*Growth temperature*

Growth temperature has been used to distinguish isolates of *Candida* species (Gales *et al*, 1999; Sancak *et al*, 2003). Failure of *C. dubliniensis* to grow on agar media at the elevated incubation temperature of 42 to 45°C has been suggested for discrimination between this species and *C. albicans*. Sancak *et al* (2003) suggested that the complete absence of growth at 42°C after 48 h of incubation, combined with an assimilation test identification as *C. dubliniensis* is strongly suggestive of this species. However, some isolates of *C. albicans* were not able to grow at 42°C (Gales *et al*, 1999) and 45°C (Tintelnot *et al*, 2000). Moreover, the poor growth at

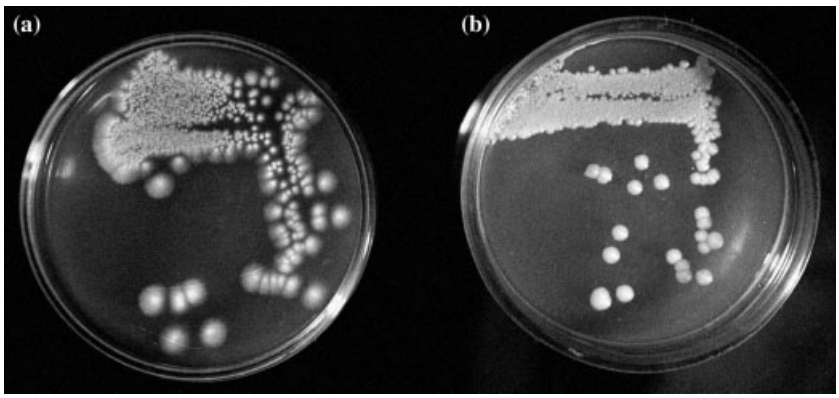


**Figure 1** Colony appearance on Niger seed (Staib) agar medium; (**a**) *C. dubliniensis*; (**b**) *C. albicans*

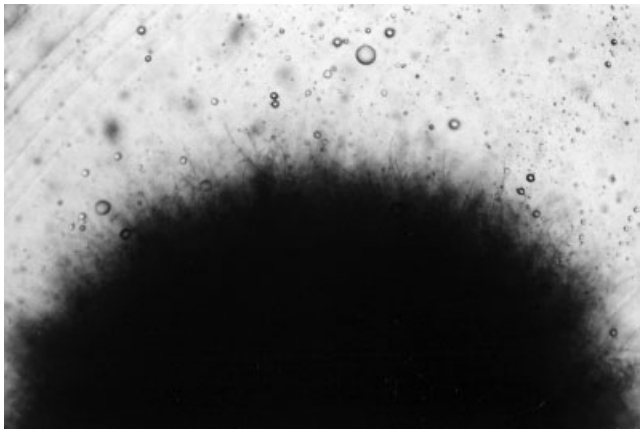

**Figure 2** Amplified colony appearance of *C. dubliniensis* on Niger seed (Staib) agar medium (magnification ×100)

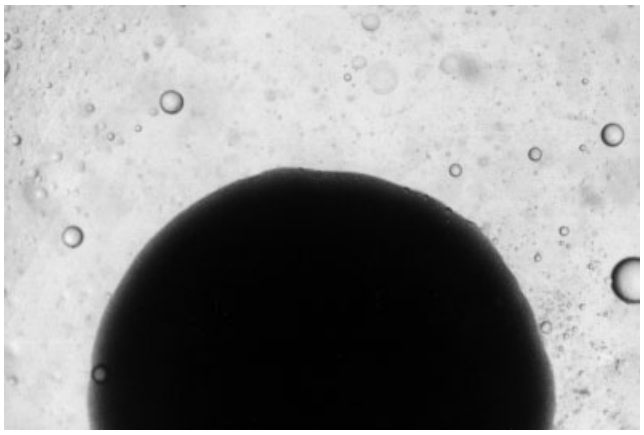

**Figure 3** Amplified colony appearance of *C. albicans* on Niger seed (Staib) agar medium (magnification ×100)

elevated temperatures is a feature of *C. dubliniensis* shared with type I *C. stellatoidea* (Coleman *et al*, 1997), which suggests that this test has a low specificity and that *C. albicans* could be falsely identified as *C. dubliniensis*.

*Coaggregation of C. dubliniensis with Fusobacterium nucleatum*

Jabra-Rizk *et al* (1999b) proposed a method to identify *C. dubliniensis* strains in clinical or research laboratories by means of coaggregation of *C. dubliniensis* with *Fusobacterium nucleatum*. After a germ tube test, a suspension of yeast cells from the original culture plate would undergo the coaggregation test with *F. nucleatum*. If no coaggregation results, *C. albicans* would be indicated, whereas coaggregation-positive isolates would be further tested as presumptive *C. dubliniensis*.

*Monoclonal antibody specific for C. albicans germ-tube test*

Marot-Leblond *et al* (2000) provided a first report on the development of a monoclonal antibody (16B1-F10), which differentiates *C. albicans* and *C. stellatoidea* from *C. dubliniensis*. This monoclonal antibody was shown by indirect immunofluorescence to be specific to the surface of the hyphal phase of the *C. albicans* and *C. stellatoidea* species whereas no labeling of *C. dubliniensis* was observed. According to the authors, efforts are being made to perfect a rapid identification test.

*Differential localization of antigens by indirect immunofluorescence test*

Bikandi *et al* (1998) developed an indirect immunofluorescence test based on differential localization of antigens on *C. dubliniensis* blastospores and *C. albicans* germ-tubes. According to the authors, the method presented evidence of the existence of antigenic differences between *C. dubliniensis* and *C. albicans*, thus providing the basis for the development of a rapid identification test.

*Hypertonic Sabouraud broth*

Alves *et al* (2002) provided the first report of a screening test for *C. dubliniensis* based on its inability to grow on Sabouraud dextrose broth with added 6.5% NaCl. The authors defined a positive test result as the lack of any visual growth of colonies in the test tube after 96 h of incubation.

*Specialized instrumental-based approaches*

Pyrolysis-mass spectrometry involves the thermal degradation of complex material (such as bacteria or fungi) to produce smaller, volatile fragments called pyrolysate. A mass spectrometer can then be used to separate the components of the pyrolysate on the basis of their mass-to-charge ratios to produce a pyrolysis mass spectrum, which can then be used as a chemical 'fingerprint' of the complex material analyzed. This technique was shown to be capable of successfully discriminating between *C. albicans*, *C. dubliniensis*, and *C. stellatoidea* (Timmins *et al*, 1998).

Fourier transform-infrared (FT-IR) spectroscopy has been used for phenotypic characterization of *C. dubliniensis* and *C. albicans* (Timmins *et al*, 1998; Peltroche-Llacsahuanga *et al*, 2000; Tintelnot *et al*, 2000). This method measures vibrations of functional groups and highly polar bonds such as O-H stretches and produces 'fingerprints' made up of the vibrational features of DNA, RNA, proteins, and membrane and cell-wall components. These 'fingerprints' are reproducible and distinct for different microorganisms. FT-IR spectroscopy combined with hierarchical clustering proved to be as reliable as genotyping for discriminating *C. albicans* and *C. dubliniensis* (Tintelnot *et al*, 2000).

The cellular fatty acid analysis by gas-liquid chromatography (GLC) analysis is an improved method for candidal discrimination (Gunasekaran and Hughes, 1980). More recently, the fatty acid methyl ester (FAME) using GLC to distinguish *C. albicans* and *C. dubliniensis*, namely Sherlock Microbial Identification System [(MIS); MIDI, Inc. Newark, DE, USA], was developed by Peltroche-Llacsahuanga *et al* (2000). Although the FAME chromatographic profiles of the two yeasts were very similar, differences in the frequency

and the amount of FAME enabled discrimination of them.

## Conclusion

*Candida dubliniensis* may be underreported in clinical samples because most currently used isolation and identification methods fail to recognize this yeast. Although some new commercial systems have incorporated *C. dubliniensis* in their databases, more studies are needed to evaluate their effectiveness. While no single phenotypic test has proven to be highly effective, two or more phenotypic methods utilized in conjunction with each other can be of value in ultimate identification of *C. albicans* and *C. dubliniensis*. A single, rapid and suitable technique for the accurate and reliable discrimination between *C. albicans* and *C. dubliniensis* has yet to be developed.

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