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Review

Current methods for identifying clinically important cryptic *Candida* species

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ABSTRACT

In recent years, the taxonomy of the most important pathogenic *Candida* species (*Candida albicans*, *Candida parapsilosis* and *Candida glabrata*) has undergone profound changes due to the description of new closely-related species. This has resulted in the establishment of cryptic species complexes difficult to recognize in clinical diagnostic laboratories.

The identification of these novel *Candida* species seems to be clinically relevant because it is likely that they differ in virulence and drug resistance. Nevertheless, current phenotypic methods are not suitable to accurately distinguish all the species belonging to a specific cryptic complex and therefore their recognition still requires molecular methods.

Since traditional mycological techniques have not been useful, a number of molecular based methods have recently been developed. These range from simple PCR-based methods to more sophisticated real-time PCR and/or MALDI-TOF methods.

In this article, we review the current methods designed for discriminating among closely related *Candida* species by highlighting, in particular, the limits of the existing phenotypic tests and the development of rapid and specific molecular tools for their proper identification.

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1. Introduction

The increasing incidence of *Candida* infections observed in recent years is the result of many factors, most notably the rise of severely immunocompromised patients (Pfaller et al., 2014). Epidemics of acquired immunodeficiency syndrome (AIDS), organ transplantation, chemotherapy treatments and other iatrogenic factors have contributed to create a population of individuals extremely vulnerable to microbial diseases in general and fungal infections in particular (Silva et al., 2012).

Predicting the clinical outcome of a systemic fungal infection is almost always a very difficult task and antifungal drug resistance is just one of many factors contributing to therapeutic failure. In fact, in addition to host-related factors (e. g. immune status, site and severity of infection), the proper identification of the disease-causing agent is also very important. However, as often happens in the clinical microbiology laboratories, some fungal species are incorrectly identified especially when automated phenotypic systems are used.

The genus *Candida* includes hundreds of species (Lachance et al., 2011) of which over 40 have been recovered from human samples (Johnson, 2009) and implicated in life-threatening infections, particularly in immunocompromised hosts.

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Candida albicans is still the most important pathogenic species of the genus although other species, which are commonly referred to as "non-albicans" *Candida* species are being increasingly isolated from clinical specimens worldwide (Brandt and Lockhart, 2012; Quindós, 2014). Among these species, *Candida glabrata*, has emerged as one of the most important opportunistic pathogens particularly able to infect a variety of human body sites (Silva et al., 2012) while *Candida parapsilosis* represents often the second most commonly isolated *Candida* species from blood cultures in many areas of the world, especially in South American and European countries and Mediterranean countries of Africa (Delfino et al., 2014; Quindós, 2014). Other species such as *Candida kefyr*, *Candida rugosa*, *Candida guilliermondii*, and *Candida famata* are rarely encountered in clinical samples although there have been several clinical case reports describing infections caused by these uncommon *Candida* species (Brandt and Lockhart, 2012; Johnson, 2009). However, the list of new pathogenic *Candida* species continues to grow since the traditional study of clinically relevant yeasts has been profoundly influenced by the development of a wide range of molecular techniques, including sequencing technologies, that combined with bioinformatics tools have allowed researchers to better understand the biodiversity, systematics and evolution of pathogenic fungi. Therefore several atypical *Candida* strains, traditionally misidentified in clinical laboratories, are now known as important pathogens of medical interest (Alcoba-Flórez et al., 2005a; Correia et al., 2006; Sullivan et al., 1995; Tavanti et al., 2005; Tietz et al., 2001).

The taxonomy of the most important *Candida* species such as *C. albicans*, *C. parapsilosis* and *C. glabrata* has undergone significant changes due to the description of new closely related species and therefore they are, now, recognized as "cryptic species complexes" (Brandt and Lockhart, 2012). Some of these novel species have also showed resistance against commonly used antifungal agents and could represent potential emerging pathogens in the future (Borman et al., 2008).

Despite the efforts made so far, there are still problems in discriminating these cryptic *Candida* species from their closest relatives (Brandt and Lockhart, 2012; Romeo and Criseo, 2011) and therefore their epidemiology, pathogenicity and clinical significance are still unclear and need more investigations.

In this article, we review the current methods designed for discriminating among closely related *Candida* species by highlighting, in particular, the limits of the existing phenotypic tests and the development of rapid and specific molecular tools for their proper identification.

2. *C. albicans* and its closest relatives

In 1995, the phylogeny of *C. albicans* underwent important changes due to the recognition of a new closely related pathogenic species called *Candida dubliniensis* (Sullivan et al., 1995). This discovery had important diagnostic consequences since this new species, like *C. albicans*, produces chlamydospores and germ-tubes and can still be misidentified as such by using biochemical tests and/or conventional identification methods (Romeo and Criseo, 2011; Yazdanpanah and Khaithir, 2014). Nevertheless, current epidemiological data showed that *C. dubliniensis* is much more prevalent in oropharyngeal infections (Wahab et al., 2014) than in invasive candidiasis (Pfaller et al., 2014) and it is rarely implicated in vaginal infections (Gumral et al., 2011; Hamad et al., 2014; Shan et al., 2014). However, *C. dubliniensis* can be easily distinguished from *C. albicans* by a number of molecular methods that have been reviewed in detail by Ells et al. (2011).

Unfortunately the most part of existing methods were specifically designed to recognize *C. dubliniensis* and/or to differentiate it from *C. albicans* and therefore they do not allow one to identify *Candida africana*, another germ-tube positive pathogenic yeast that was proposed as a new *Candida* species in 2001 (Tietz et al., 2001).

C. africana was first isolated from female genitals from African patients and, as for *C. dubliniensis*, it was initially considered an atypical *C. albicans* strain with unusual phenotypes (Romeo et al., 2013a). In fact, all *C. africana* isolates reported so far formed germ-tubes in serum but they failed to produce chlamydospores and were also unable to assimilate a number of carbon sources including N-acetylglucosamine and glucosamine (Tietz et al., 2001), two monosaccharides utilized in the order of 100% and 99–100% by *C. albicans* strains. Nevertheless, although *C. africana* shows a number of phenotypic characteristics clearly different from those of typical *C. albicans* and *C. dubliniensis* isolates (Romeo and Criseo, 2011), its identification is still difficult using routine tests currently available in clinical diagnostic laboratories (Romeo and Criseo, 2009a). In reality, as reported for *C. dubliniensis* (Lockhart, 2011), differentiation of *C. africana* from *C. albicans* seems to be not clinically relevant but recent studies highlighted a greater role of *C. africana* in genital infections (Romeo et al., 2013a) and denote *C. dubliniensis* as an important bloodstream pathogen (Khan et al., 2012a). Therefore the correct identification of the members of the *C. albicans* species complex appears to be an essential prerequisite to better understand the epidemiology, clinical significance and evolution of antifungal resistance of these microorganisms.

Today, several automated identification systems such as VITEK 2 YST and/or ID 32C (bioMérieux, Marcy l'Etoile, France) have included the biochemical profiles of *C. dubliniensis* in their respective databases and therefore the recognition of this species in clinical laboratories is more easy (Albaina et al., 2015; Alves et al., 2005; Melhem et al., 2014). Recent data, in fact, showed that the ID 32C system makes also possible the identification of unusual clinical isolates of *C. dubliniensis* (Albaina et al., 2015) and allows a reliable differentiation of *C. africana* on the basis of its characteristic assimilation pattern (Romeo and Criseo, 2011; Tietz et al., 2001). In addition, *C. dubliniensis* isolates can be differentiated by *C. albicans* using a number of simple phenotypic screening tests including their capacity to produce rough colonies and abundant chlamydospores on Staib agar, inability to grow in hypertonic Sabouraud broth (6,5% NaCl) and in agar media containing xylose as the sole carbon source (Ells et al., 2011; Khan et al., 2012b). Unfortunately these methods often give ambiguous results and most importantly they do not allow one to recognize *C. africana* which is misidentified as *C. albicans*. In fact *C. africana* showed a low filamentation rate (Romeo et al., 2011), no chlamydospores and it is able to assimilate xylose and grow in hypertonic broths (Romeo et al., 2013a; Romeo and Criseo, 2011; Tietz et al., 2001). Interestingly, phenotypic misidentification may also occur between *C. africana* and some atypical *C. dubliniensis* isolates that are unable to form chlamydospores (Albaina et al., 2015). In fact, a very recent study reported the existence of two atypical populations of *C. dubliniensis* with ambiguous phenotypic characters, which further complicate the discrimination within the *C. albicans* complex (Albaina et al., 2015). Group I isolates were unable to form chlamydospores and germ-tubes and did not react using a monoclonal antibody highly specific for *C. dubliniensis* while group II isolates showed pink and white colonies on CHROMagar *Candida* (CHROMagar, Paris, France) and ChromID *Candida* (bioMérieux, Marcy l'Etoile, France) where, usually, *C. dubliniensis* grow forming dark green and turquoise colonies respectively (Albaina et al., 2015). In line with these observations, which emphasize the need to simultaneously use different phenotypic tests for presumptive identification of all members of the *C. albicans* complex, we propose a general scheme for basic initial screening of these yeast isolates (Table 1). However, species identification based on phenotypic methods is not always sufficient and is often influenced by culture conditions that may vary from one laboratory to another. For these reasons, to confirm the identity of the isolated species, phenotypic tests have to be supplemented with molecular-based methods (Li et al., 2014; Nnadi et al., 2012; Romeo et al., 2012; Yazdanpanah and Khaithir, 2014).

Currently, only few molecular methods have been reported for specific identification of *C. africana* (Table 2). One of these, has been widely used in several epidemiological studies (Albaina et al., 2015; Gumral et al., 2011; Ngouana et al., 2015; Nnadi et al., 2012; Romeo and