Comparison between multiplex PCR and phenotypic systems for *Candida* spp. identification

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SUMMARY

This study evaluated the performances of three phenotypic systems (RapID Yeast panel, Vitek2 YST card, and API 20 C AUX) and multiplex PCR for *Candida* spp. identification.
Four-hundred and fifty clinical strains of *Candida* spp. were identified with the four systems and results of multiplex PCR were compared with those of phenotypic methods. The best correspondence was obtained between Multiplex PCR and API 20 C AUX (83.7%), but the other comparisons showed similar values (81.7% and 79.3% for Vitek2 and RapID Yeast panel respectively). The correspondence was lower for all the methods in identification of *C. krusei*; this may be of concern in addition to the azole resistance and the often endogenous origin of this yeast. In the comparison with the three phenotypic methods, multiplex PCR could be reliable and time-saving in the identification of *Candida* spp. for diagnostics purposes.
Nowadays, a large variety of both traditional and molecular methods for *Candida* spp. identification are commercially available. Multiplex PCR applied in this study may be more rapid and sensitive than phenotypic systems, and less expensive than other molecular methods.

KEY WORDS: Candida spp., PCR, Phenotypic systems

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INTRODUCTION

Yeasts can cause a spectrum of diseases ranging from superficial manifestations to systemic and severe infections. Although *Candida albicans* is still the most common cause of fungal infections, the emergence of non-*albicans Candida* spp. has been observed worldwide (Fridkin and Jarvis, 1996; Hobson, 2003).
Diagnostic strategies allowing rapid and accurate identification of isolates may be helpful to study their epidemiology, spread and routes of transmission and to address therapeutic choices (White *et al.,* 1998).
Nowadays, a large variety of identification methods are commercially available, and they differ in principles, discriminatory power and costs. Traditional microbiological procedures are based on macroscopic and microscopic analysis of colonies and on the biochemical characteristics of the yeasts (Freydiere *et al.,* 2001). In the last decade, several molecular methods have been developed for the identification of *Candida* spp. and they appear reliable and easy to use (Chang *et al.,* 2001; Luo and Mitchell, 2002).
This study compared a multiplex PCR method tested in previous experiments with three phenotypic *Candida* identification systems (Liguori *et al.,* 2007; Liguori *et al.,* 2009).

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MATERIALS AND METHODS

The study was carried out in the years 2005-2007. A collection of 450 *Candida* strains was obtained by routine isolation from different clinical materials in the laboratories of the Hygiene Section, Department of Public, Clinical and Preventive Medicine of the Second University of Naples and of the Microbiology Section, "L. Califano" Department of Cellular and Molecular Biology and Pathology.

First cultures were obtained on Sabouraud-Dextrose-Agar (SDA) + chloramphenicol after 48 h at 37°C. Each isolate was identified by API 20 C AUX (bioMérieux S.p.A.) and stored at -70°C in glycerol; one aliquot was then subcultured as reported for the first isolation and used for identification through multiplex PCR, RapID Yeast Identification panel - RYIP - (Dade Behring S.p.A.) and Vitek2 YST card (bioMérieux S.p.A.), and for a new identification with API 20 C AUX, together with the reference ATCC strains *C. albicans* 90028, *C. albicans* 36082, *C. parapsilosis* 22017, *C. krusei* 6258, *C. neoformans* 1199 and *C. neoformans* 6852. All the procedures were performed as recommended by the manufacturers.

Multiplex PCR

The amplification reaction was carried out directly on the colony suspension, without DNA extraction, as previously reported (Chang *et al.,* 2001; Liguori *et al.,* 2007; Liguori *et al.,* 2009). The method amplified the internal transcribed spacer 1 (ITS1) region between the 18S and 5.8S rRNA genes and a portion of the ITS2 region of *Candida albicans*.

Briefly, we added a colony from fresh culture to a 50 µL reaction mix containing Taq Buffer 10 ×, dNTPs 10 mM, primers 5 µM and 0.5 µL Taq pol 5 u µL-1; the primers were ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3'), ITS2 (5'-GCT GCG TTC TTC ATC GAT GC-3'), CA3 (5'-GGT TTG CTT GAA AGA CGG TAG-3'), CA4 (5'-AGT TTG AAG ATA TAC GTG GTA G-3'), ITS1F (5'-CCA GCG CTT AAT TGC G-3'), ITS1K (5'-ATC GTC TGA ACA AGG CCT GC-3') and ITS2D (5'-GAG AAC CAA GAG ATC CGT TGT TG-3'). The amplicon lengths are reported in Table 1.

The mix was then submitted to a predenatura-

TABLE 1 - *Length of PCR products.*

Species	*Primers*							*PCR product length (bp)*		
	ITS1	*ITS1F*	*ITS1K*	*ITS2*	*ITS2D*	*CA3*	*CA4*			
C. albicans	+	-	-	+	+	+	+	218-219	198-199	110
C. glabrata	+	-	-	+	+	-	-	482-483	462-463	-
C. guilliermondii	+	-	-	+	+	-	-	248	228	-
C. famata	-	+	-	+	+	-	-	234	214	-
C. kefyr	-	-	+	+	+	-	-	249	229	-
C. parapsilosis	+	-	-	+	+	-	-	229	209	-
C. tropicalis	+	-	-	+	+	-	-	218	199	-
C. krusei	+	-	-	+	+	-	-	182	166	-
C. lusitaniae	+	-	-	+	+	-	-	148	128	-
C. dubliniensis	+	-	-	-	+	-	-	-	198	-
S. cerevisiae	+	-	-	+	-	-	-	840	-	-
C. incospicua	+	-	-	+	-	-	-	460	-	-

tion at 95°C for 10 min, 40 cycles of 1 min at 95°C, 1 min at 50°C, 1 min at 72°C and a final extension at 72°C for 10 min. The presence and the length of the amplicons were analyzed in a 2% agarose gel with ethidium bromide (0.5 µg mL-1). The primers allows the identification of *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. krusei*, *S. cerevisiae*, *C. parapsilosis*, *C. guillermondii*, *C. kefyr*, *C. famata*, *C. dubliniensis*, *C. lusitaniae*, *C. incospicua* (Liguori *et al.,* 2007; Liguori *et al.,* 2009). On the basis of the routine identification results, we enrolled in this study only the isolates belonging to these species.

Processing results

To determine the level of concordance between the analyzed systems, the comparison was performed by evaluating the number of phenotypic identifications corresponding to those of the molecular method for each species. Only the results with a good discrimination were considered.

RESULTS

The reference strains were correctly identified by all the methods (data not shown). The identifications obtained with the four systems and the number of those corresponding between PCR and each phenotypic system per species are reported in Table 2, together with the percentages of concordance.

Multiplex PCR showed better correspondence with the API 20 C AUX (83.7%), although the other systems gave similar values (81.7% and 79.3% for Vitek2 and RapID Yeast panel respectively). However, many discrepancies were also recorded in the identification of *C. albicans*, which is the most frequently isolated species, and also in the comparison between PCR and the API system (which misidentified 34 *C. albicans* as 15 *C. tropicalis*, 7 *C. glabrata*, 3 *C. parapsilosis*, 3 *C. krusei*, 2 *C. kefyr*, 2 *C. maris*, 1 *C. famata*, 1 *C. lusitaniae*). Regarding Rapid Yeast Plus panel, 33 *C. albicans* were misidentified as *C. glabrata* (13), *C. parapsilosis* (8), *C. tropicalis* (5), *C. kefyr* (2), *C. krusei* (1), *S. cerevisiae* (1), *C. neoformans* (2) and *Pichia farinosa* (1). Instead, 46 *C. albicans* strains were misidentified from Vitek2 as *C. glabrata* (12), *C. parapsilosis* (9), *C. tropicalis* (6), *C. norvegensis* (4), *C. dubliniensis* (3) *C. famata* (3), *C. krusei* (2), *C. rugosa* (2), *C. sake* (1), *C. colliculosa* (1) and *C. sphaerica* (1).

TABLE 2 - *The first column gives the number of strains identified by PCR for each species. The other columns show the number (% in parentheses) of identification per species concordant with PCR. The last row gives concordance values (%).*

	PCR	*API 20C AUX*	*RYIP*	*VITEK2*
C. albicans	261	227(87)	228 (87)	215 (82)
C. glabrata	90	78 (86)	62 (69)	69 (77)
C. tropicalis	24	18 (75)	18 (75)	17 (71)
C. krusei	9	5 (55)	2 (22)	6 (67)
S. cerevisiae	16	14(87)	14 (87)	11(69)
C. parapsilosis	28	21 (75)	24 (86)	26 (93)
C. guilliermondii	3	2 (67)	0	3 (100)
C. kefyr	8	7 (87)	7 (87)	3 (37)
C. famata	1	1 (100)	0	0
C. dubliniensis	2	0	0	2 (100)
C. lusitaniae	4	3 (75)	0	4 (100)
C. inconspicua	1	1 (100)	1 (100)	0
Concordance values		83,7%	79,3%	81,7%

DISCUSSION

The accurate identification of yeasts is a fundamental goal of microbiology laboratories, mainly in hospital settings and for critical patients (Chen *et al.,* 2002; Erjavec and Verweij, 2002).

In the last decade several techniques have been developed to improve this practice and many molecular methods being used with phenotypic tests in this effort. Recent commercial introductions include several chromogenic media, direct enzymatic tests, automated biochemical and enzymatic panels and immunological methods.

In our past experiments we tested a protocol of Multiplex PCR for the identification of strains be-

longing to the 12 main species of *Candida* causing oral candidiasis. To reduce the time for identification, the PCR was also applied directly on the sputum samples. In both cases the method proved accurate and simple. In a comparison with phenotypical methods, differences were observed between PCR analysis on colonies and chromogenic media; similar differences were observed in the case of Vitek 2. Conversely, no discordance was seen with the API system or with RYIP (Liguori *et al.,* 2007; Liguori *et al.,* 2009).
As previously reported, PCR analysis without preliminary DNA extraction detected yeasts even with mycotic counts as low as 10 cfu ml-1 and in samples heavily contaminated by bacteria (Liguori *et al.,* 2007) and it does not require toxic and expensive chemical reagents (Carvalho *et al.,* 2007; Liguori *et al.,* 2007; Liguori *et al.,* 2009). Here we extended the use of PCR analysis to the identification of a large number of strains isolated from different clinical samples, and we compared their results with those supplied by three commercial phenotypic systems for *Candida* spp. identification: API 20 C AUX, Vitek2 and RapID Yeast panel.
The API 20 C AUX is today considered a reference method among phenotypic tests (Asticcioli *et al.,* 2009; Aubertine *et al.,* 2006; Hata *et al.,* 2007). It requires precision during test implementation, experience in the interpretation of turbidity level and sometimes long incubation times, but it is reliable, easy to use and inexpensive. However, it is impossible to identify *C. dubliniensis* with this method.
The Vitek 2 ID-YST system and the RapID Yeast Plus panels are quite precise in the identification, of all the species (Erjavec and Verweij, 2002; Guelfand *et al.,* 2003; Heelan *et al.,* 1998; Smith *et al.,* 1999), and Vitek 2 can correctly identify common and also uncommon yeasts, including *C. dubliniensis* (Freydiere *et al.,* 2001). They are also less time-consuming than API system. However, they require automatized equipments (colorimetric Vitek2 system and spectrophotometer for the RYIP) which can be expensive.
For all the phenotypic methods, the performances strictly depend on inoculum density and sometimes there is a need for additional tests to obtain a definitive result.
Here the comparison between multiplex PCR and the API system showed the best concordance level, although the other phenotypic methods gave similar results.
There was a certain variability among phenotypic methods in the identification of some species (i.e. *C. glabrata* or *C. krusei*), and more misidentifications were recorded for RYIP and Vitek 2 with regard to the most commonly isolated species (i.e. *C. tropicalis*, *C. glabrata*, *C. krusei*).
Lastly, the concordance with PCR in the identification of *C. krusei* was lower for all the other methods. This may be a concern in the treatment of infections caused by this species, which is already difficult because of intrinsic azole resistance and endogenous origin frequently associated with this yeast, which is often responsible for recurrent episodes of infection (Agirbasli *et al.,* 2008; Asticcioli *et al.,* 2009; Pfaller *et al.,* 2008). The use of molecular methods, especially with species-specific primers/probes, is advisable in these circumstances.
When specific molecular biology equipment and trained personnel are lacking, the phenotypic systems remain the first choice for yeast identification. However, although they are reliable, the methods tested in this experiment require a longer time for identification than the molecular method. The possibility to carry out yeasts detection and identification directly on the clinical sample, without nucleic acid extraction and eliminating the time for culture, together with the reduction of costs, represent some of the main goals of microbiological clinical diagnostics. In this perspective, multiplex PCR can represent a valid solution. The availability of more primers could potentially allow the identification of all the species of *Candida* with the accuracy of a genetic system and without the costs of other molecular methods (i.e. sequencing), which limit their widespread use.

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