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ORIGINAL ARTICLE

Candida clinical species identification: molecular and biochemical methods

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Abstract In the last decade, the number and diversity of nosocomial *Candida* infections has increased significantly, resulting in an emergent need for rapid and accurate methods for *Candida* identification. Therefore, the aim of this study was to evaluate the performance of three biochemical systems (Auxacolor, ID32C, and Vitek 2 YST) for the identification of *Candida* species, comparing them with molecular identification (polymerase chain reaction and gel agarose electrophoresis). These methods were used to assess *Candida* spp. (229 clinical isolates) prevalence and distribution among clinical specimens. The biochemical methods with higher percentages of correct identification were Vitek 2 YST (79.6%) and Auxacolor (78.6%). However, overall the biochemical methods assayed differed from the molecular identification. Thus, due to their rapid and precise identification, molecular methods are promising techniques for *Candida* species identification in clinical laboratories. *Candida albicans* and Non *Candida* albicans *Candida* species had a similar prevalence (50.4 and 49.6%, respectively), corroborating the epidemiological shift observed for these pathogens in the recent years.

Keywords *Candida* species · Discriminating potential · PCR · Vitek 2 YST · Auxacolor · ID32C

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Introduction

In the last two decades, the incidence of *Candida* infections, associated with high morbidity and mortality (Goodrich et al. 1991; Beck-Sagué and Jarvis 1993; Karlowsky et al. 1997; Nucci et al. 1997), has increased remarkably (Warren and Hazen 1995; Wingard 1995; Ribeiro et al. 2004). Although *Candida albicans* is the most prevalent species (Fridkin and Jarvis 1996), an epidemiological shift of *Candida* pathogens has been recently denoted by the increasing number of infections caused by non-*Candida albicans Candida* (NCAC) species (Hazen 1995; Jarvis 1995; Fridkin and Jarvis 1996; Nauyen et al. 1996). This increased species diversity and incidence of infections resulted in the need for rapid and accurate methods for *Candida* identification (Verweij et al. 1999; Wadlin et al. 1999; Hsu et al. 2003; Crocco et al. 2004). This will have a decisive contribution in the evolution of treatment by adequate antifungal selection, and allow the epidemiologic study of colonization and infection in hospitals (Campbell et al. 1999; Verweij et al. 1999; Ribeiro et al. 2004). Several commercial systems have been developed and compared for yeast identification (Fenn et al. 1994; Gutierrez et al. 1994; Fricker-Hidalgo et al. 1995; Verweij et al. 1999; Hata et al. 2007), such as the manual Auxacolor (colorimetric) and ID32C (turbidimetric) systems, and the automated Vitek 2 YST (colorimetric) system (Campbell et al. 1999; Verweij et al. 1999). Table 1 shows a brief review of the comparison of the methods found in the literature.

Misidentifications between some *Candida* species are frequently observed, particularly between *C. dubliniensis* and *C. albicans* (Abaci et al. 2008). To distinguish between them, methods such as the latex agglutination test can be used (Kurzai et al. 1999; Marot-Leblond et al. 2006).

However, in spite of the advances in biochemical commercial systems, so far they do not allow for the rapid diagnosis of

Table 1 Brief review of method comparisons in the literature

Authors	Year	Compared methods	Conclusions
Fenn et al.	1994	Vitek Yeast Biochemical Card (YBC); API 20C	Vitek YBC compared favourably with the API 20C in the identification of common yeasts. However, Vitek demonstrated problems in the identification of *C. tropicalis*, *C. krusei*, *Trichosporon* spp., and some *Cryptococcus* spp.
Fricker-Hidalgo et al.	1995	Auxacolor; ID 32C	Auxacolor was found to be reliable when performed in conjunction with morphological tests. Furthermore, Auxacolor provided faster results than ID 32C.
Verweij et al.	1999	Vitek; ID 32C; Api 20C AUX; Yeast Star; Auxacolor; RapID Yeast Plus system; Api Candida; Conventional tests	Auxacolor and Api Candida appeared to be the most useful systems for identification of germ tube negative yeasts.
Wadlin et al.	1999	RapID Yeast Plus System; API 20C Aux; Vitek Yeast Biochemical Card, microscopic morphologic reference method	The RapID Yeast Plus System was significantly better than the other methods. There was no significant difference between results obtained with API 20C Aux and Vitek Yeast Biochemical Card.
Campbell et al.	1999	API Candida; Auxacolor	The methods had similar percentages of correct identification. However, the API Candida system required supplemental biochemical tests or morphological assessment to obtain the correct identification, and gave more incorrect identifications.
Kanbe T et al.	2003	PCR amplification; Conventional identification techniques	The results of identification of clinical samples based on the PCR amplification coincided with those of conventional identification techniques.
Liguori et al.	2007	Multiplex PCR assay; Routine phenotypic culture identification	The multiplex PCR assay provides a rapid alternative to the conventional culture based technique for the identification and speciation of the most frequently isolated Candida species.

invasive fungal infections (Goodwin et al. 1992; Hazen 1995; Espinel-Ingroff et al. 1998), leading to a late introduction of the specific antifungal therapy (Goodwin et al. 1992; Hazen 1995; Espinel-Ingroff et al. 1998; Hsu et al. 2003; Huang et al. 2006). In this sense, molecular biology techniques, such as PCR, might be more accurate and efficient, due to their reproducibility, high specificity, and sensitivity (Hidalgo et al. 2000; Kanbe et al. 2002, 2003; Hsu et al. 2003), allowing same day identification. Nevertheless, their lack of standardization for *Candida* species identification, as well as the extra costs associated and the reliance on *Candida* cultures that results in a considerable delay in comparison to direct specimen detection, may limit its use.

The aim of this study, carried out at the Service of Clinical Pathology of Hospital de São Marcos (HSM), was to evaluate the performance of the biochemical systems Auxacolor, ID32C, and Vitek 2 YST for the identification of *Candida* species, comparing to a molecular identification, and to assess *Candida* spp. prevalence and distribution among clinical specimens.

Materials and methods

Clinical isolates and culture conditions A total of 229 isolates were randomly isolated from clinical samples (sputum/bronchial wash/bronchoalveolar lavage, vaginal swab, blood culture, catheter tip and urine, among others) between December 2005 and March 2007, in HSM. The clinical isolates were kept in Brain Heart Infusion (BHI, 37 g/l; Bio-Rad, Marnes-la-Coquette, France) with 10% glycerol (Vaz Pereira, Lisbon, Portugal), at -70°C. Prior to testing, isolates were retrieved from storage and subcultured, for 24 to 48 h, on plates of Columbia sheep blood agar (bioMérieux, Lyon, France).

Reference strains For quality control, ATCC strains were used: *Candida albicans* ATCC 90028, *C. albicans* ATCC 14053, *Candida glabrata* ATCC 2001, *Candida tropicalis* ATCC 750, *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 (MicroBiologics, Saint Cloud, MN, USA); and a *Candida dubliniensis* strain provided by Biognóstica from United Kingdom National External Quality Assessment Service. These strains were maintained and grown as described for clinical isolates.

Biochemical identification

Auxacolor system The yeast identification procedures were performed according to the manufacturer's instructions (Bio-Rad). The results were read after incubation for 24 h

and 48 h, and identifications were made by referring to the list of numerical profiles provided by the manufacturer.

ID32C system All tests were performed according to the manufacturer's instructions (bioMérieux). The results were read automatically after incubation for 24 and 48 h using the *mini API* system (bioMérieux).

Vitek 2 YST system The yeast identification procedures were performed according to the manufacturer's instructions (bioMérieux). Cards were automatically read, and after approximately 18 h a final identification was obtained using the Vitek 2 database.

Latex aglutination test Bichro-Dubli Fumouze latex agglutination test (Fumouze Diagnostics, Levallois-Perret Cedex, France) was performed to identify *C. dubliniensis* strains. This test is based on the coagglutination of *C. dubliniensis* blastoconidia with blue latex particles coated with a monoclonal antibody, allowing the specific detection of the *C. dubliniensis* cell-surface antigen. All tests were performed according to the manufacturer's instructions.

Molecular identification

DNA purification A 100 µl yeast suspension was prepared in aqueous saline solution 0.45% (w/v) NaCl with a turbidity equivalent to 2.0 McFarland. DNA was extracted, isolated and purified according to the protocol of Magna Pure LC DNA Isolation Kit III (Roche Applied Science, Amadora, Portugal). Purified DNA was preserved at −20°C.

Nested PCR DNA samples were amplified in a reaction mixture (50 µl) that contained 5 µl genomic DNA, 17 µl double distilled water (ddH_2O; Roche Applied Science), 1.5 µl of each degenerated primer (Table 2) targeting *Candida* DNA topoisomerase II gene, which were chosen

Table 2 Primers used for PCR amplification, oligonuclotide sequences and fragment size

Primer and target species	Name	Sequence (5′–3′)	Amplified fragment size (bp)
Degenerated primers	CDF28	GGTGGWMGDAAYGGDTWYGGYGC	1200
	CDR148	CCRTCNTGATCYTGATCBGYCAT	
Specific primers			
Candida albicans	CABF59	TTGAACATCTCCAGTTTCAAAGGT	515
	CABR110	GTTGGCGTTGGCAATAGCTCTG	665
	CADBR125	AGCTAAATTCATAGCAGAAAGC	
Candida dubliniensis	CDBF28	AAATGGGTTTGGTGCCAAATTA	816
	CDBR110	GTTGGCATTGGCAATAGCTCTA	966
	CADBR125	AGCTAAATTCATAGCAGAAAGC	
Candida tropicalis I	CTPIF36	GTTGTACAAGCAGACATGGACTG	318
	CTPIR68	CAAGGTGCCGTCTTCGGCTAAT	853
	CTPIR121	TCAAGGTACAGTTATGGCCAAGTT	
Candida tropicalis II*	CTPIIF36	CTGGGAAATTATATAAGCAAGTT	246
	CTPII60	CTTGAGATACTCAATCTTTTATC	860
	CTPIIR121	TCAATGTACAATTATGACCGAGTT	
Candida parapsilosis I	CPPIF41	TGACAATATGACAAAGGTTGGTA	228
	CPPIR61	ACTTTTAAAACTGTTAACCGA	837
	CPPIR122	TGTCAAGATCAACGTACATTTAGT	
Candida parapsilosis II[a]	CPPIIF41	GGACAACATGACAAAAGTCGGCA	310
	CPPIIR69	TTGTGGTGTAATTCTTGGGAG	837
	CPPIIR122	GGTAAGGATCAAAGTGCACTTTA	
Candida krusei	CKSF35	GAGCCACGGTAAAGAATACACA	227
	CKSR57	TTTAAAGTGACCCGGATACC	756
	CKSR110	TTTCTCTGGCAATTCCAATCG	
Candida glabrata	CGBF35	CCCAAAAATGGCCGTAAGTATG	419
	CGBR77	CTGCTTGAAAGAAATATCGGAGAC	674
	CGBR103	ATAGTCGCTACTAATATCACACC	

[a] These species have two distinct genotypes in the DNA topoisomerase gene[12]

according to Kanbe et al. (2002), and 25 μl PCR Master Mix (Roche Applied Science). The PCR was performed in a thermocycler (T1 Model; Biometra, Goettingen, Germany) with the following cycle parameters: preheating at 94°C for 2 min, 40 cycles at 94°C for 30 s, 55°C for 20 s, and 72°C for 90 s. These PCR products were further amplified by species specific primers (Table 2), also targeting *Candida* DNA topoisomerase II gene (Kanbe et al. 2002), in a reaction mixture containing 19.5 μl ddH_2O, 1.5 μl of each species specific primers, 25 μl PCR Master Mix, and 1 μl DNA template. The PCR thermocycler parameters were the same as described above.

Agarose gel electrophoresis Detection of PCR products was performed by electrophoresis (MiniRun GE-100; Bioer, Tokyo, Japan) on a 1.2% agarose (QA-Agarose TM; MP Biomedicals, Illkirch, France) gel prepared in 0.5X Tris-Borate-EDTA (TBE; Severn Biotech, Worcestershire, UK), at 100 V for 35 min in TBE. Gels were stained with ethidium bromide (10 mg/ml; Molecular Sigma Biology, Sintra, Portugal) for 20 min, and destained in TBE for 10 min. DNA bands were visualized with a UV transilluminator (Model TM-15E Chromato-Vue®; UVP, Cambridge, UK) and photographed. PCR product band size was determined by comparison with a 100 bp DNA ladder (Zymo Research, Orange, CA, USA) ran in the same gel.

Methodology for the analysis of the identification results The biochemical systems of identification were evaluated by comparison with the molecular method (assumed as the reference). Since only the specific primers for *C. albicans*, *C. parapsilosis*, *C. tropicalis*, *C. glabrata*, *C. krusei*, and *C. dubliniensis* were acquired, just 196 isolates were compared. Biochemical identification was understood as: correct when identical to the molecular results; incomplete when it gave more than one possibility of identification (including the correct); incorrect when different from the molecular results; and unidentified when the test gave no identification at all.

Statistical analysis Data from *Candida* species identification were statistically analyzed with Statistical Package for Social Sciences (SPSS), using the Cochran's Q and McNemar tests with a confidence level of 95%.

Results

Biochemical and molecular identification

Table 3 summarizes the identification results obtained by the biochemical methods for *Candida* species, in comparison with the molecular identification. The reference strains were correctly identified by all methods, except for *C. glabrata* 2001 and *C. krusei* 6258 with Auxacolor and *C. krusei* 6258 with Vitek 2 YST, where incomplete identifications were achieved. For the clinical isolates, *C. parapsilosis* strains were the species most correctly identified by the biochemical methods, while *C. glabrata* and *C. krusei* were the species with lower levels of correct identification.

Overall, Vitek 2 YST and Auxacolor (48 h incubation) gave the higher percentages of correct identification (79.6 and 78.6%, respectively).

Results of the biochemical and molecular identification were firstly statistically analyzed with the Cochran's Q test. As the significance value (P=0.000) indicated significant differences among the methods, their responses were subsequently analyzed with the McNemar test. It was possible to verify that ID32C results diverged significantly from the other biochemical methods, being the method with worst results for *Candida* species identification. Furthermore, it was observed that the incubation time is an important aspect for the Auxacolor method, where significantly better results are achieved after 48 h incubation.

Results from biochemical methods were also compared with molecular identification, as shown in Table 4. All the biochemical methods provided significantly divergent results from molecular identification, with the exception of Auxacolor (48 h incubation) and ID32C (24 h incubation) for *C. parapsilosis*.

Latex agglutination test

The agglutination test, performed for strains identified as *C. albicans* or *C. dubliniensis* by biochemical methods, provided a positive result for one strain, identified as *C. dubliniensis* by molecular methods, indicating its accuracy.

Prevalence and distribution of *Candida* species

Figure 1 shows the prevalence and distribution of *Candida* species among clinical specimens. *Candida albicans* was the most prevalent species (50.4%), followed by *C. parapsilosis* (13.9%), *C. tropicalis* (10.5%), *C. glabrata* (9.1%), *C. krusei* (0.9%), *C. dubliniensis* (0.4%) and other unidentified *Candida* species (14.8%). *Candida* species were more frequently isolated from clinical specimens of urine (38.9%), sputum/bronchial wash/bronchoalveolar lavage (24.9%), and vaginal swabs (11.4%). The second was also the specimen where a major diversity of species was encountered. Indeed, the only strain of *C. dubliniensis* and the two strains of *C. krusei* identified were isolated from sputum/bronchial wash/bronchoalveolar lavage. It should also be noted that *C. glabrata* was isolated in higher

Table 3 Results of the evaluation of 196 strains using the Auxacolor, ID 32C, and Vitek 2 YST systems, assuming the molecular method as reference

Species	System and ncubation time (h)	Isolates with the following identification (%)			
		Correct	Incomplete	Incorrect	Unidentified
Candida albicans (*n*=116)	Auxacolor				
	24	82.8	0.9	2.6	13.8
	48	92.2	0.9	2.6	4.3
	ID32C				
	24	68.1	0.9	0.9	30.2
	48	69.0	1.7	0.0	29.3
	Vitek 2 YST	87.1	7.8	3.4	1.7
Candida parapsilosis (*n*=32)	Auxacolor				
	24	62.5	9.4	21.9	6.3
	48	93.8	3.1	0.0	3.1
	ID32C				
	24	84.4	0.0	0.0	15.6
	48	75.0	0.0	0.0	25.0
	Vitek 2 YST	78.1	15.6	3.1	3.1
Candida tropicalis (*n*=24)	Auxacolor				
	24	58.3	4.2	12.5	25
	48	58.3	4.2	8.3	29.2
	ID32C				
	24	50.0	8.3	4.2	37.5
	48	66.7	0.0	4.2	29.2
	Vitek 2 YST	75.0	0.0	4.2	20.8
Candida glabrata (*n*=21)	Auxacolor				
	24	4.8	90.5	4.8	0.0
	48	9.5	76.2	4.8	9.5
	ID32C				
	24	42.9	0.0	0.0	57.1
	48	52.4	0.0	0.0	47.6
	Vitek 2 YST	52.4	14.3	4.8	28.6
Candida krusei (*n*=2)	Auxacolor				
	24	0.0	100	0.0	0.0
	48	0.0	100	0.0	0.0
	ID32C				
	24	0.0	100	0.0	0.0
	48	0.0	100	0.0	0.0
	Vitek 2 YST	0.0	50.0	0.0	50.0
Candida dubliniensis (*n*=1)	Auxacolor				
	24	100	0.0	0.0	0.0
	48	100	0.0	0.0	0.0
	ID32C				
	24	0.0	0.0	100	0.0
	48	0.0	0.0	100	0.0
	Vitek 2 YST	100	0.0	0.0	0.0
Candida (total) (*n*=196)	Auxacolor				
	24	67.3	13.3	7.1	12.2
	48	78.6	10.7	3.1	7.7
	ID32C				
	24	64.8	2.6	1.5	31.1
	48	66.8	2.0	1.0	30.1
	Vitek 2 YST	79.6	9.2	3.6	7.7

Table 4 Significance values (*P*) obtained from the comparison between the results of biochemical systems and the molecular method

System and incubation time	Significance (*P*)				
	Candida albicans	*Candida parapsilosis*	*Candida tropicalis*	*Candida glabrata*	*Candida* (overall)
Auxacolor 24 h	0.000	0.000	0.002	0.000	0.000
Auxacolor 48 h	0.004	0.500	0.002	0.000	0.000
ID32C 24 h	0.000	0.063	0.000	0.000	0.000
ID32C 48 h	0.000	0.008	0.008	0.002	0.000
Vitek 2 YST	0.000	0.016	0.031	0.002	0.000

numbers from vaginal swabs, and *C. parapsilosis* from the group identified as "Other".

Discussion

In this work, three biochemical systems were evaluated (Auxacolor, ID32C, and Vitek 2 YST) in comparison to molecular identification. Considerable differences were noted between the biochemical and molecular identification, especially for ID32C, which was demonstrated to be the less accurate system. These divergences were observed for all *Candida* species, with the exception of *C. parapsilosis* for Auxacolor (48 h incubation) and ID32C (24 h incubation). Indeed, this species was the easiest to identify by biochemical methods, followed by *C. albicans*, whose levels of correct identification were high for Auxacolor and Vitek 2 YST. On the other hand, *C. glabrata* and *C. krusei*, usually associated with high levels of antifungal resistance, posed the greatest identification problems. For *C. krusei* it is not possible to reach any conclusion since the number of isolates was too low (two). As for *C. glabrata*, it could be said that it is the hardest species to identify, especially by Auxacolor whose higher percentages of incomplete identification should be emphasized. *Candida tropicalis*, although not with the extension of *C. glabrata*, was also difficult to identify with ID32C and Auxacolor.

Regarding *C. dubliniensis*, since the number of strains identified (one) did not allow a statistical analysis of its identification, it can merely be stated that Vitek 2 YST and Auxacolor correctly identified the isolate, and ID32C identified it, incorrectly, as *C. albicans*. Indeed, the distinction between these two species is problematic. Therefore, a latex agglutination test was performed, which

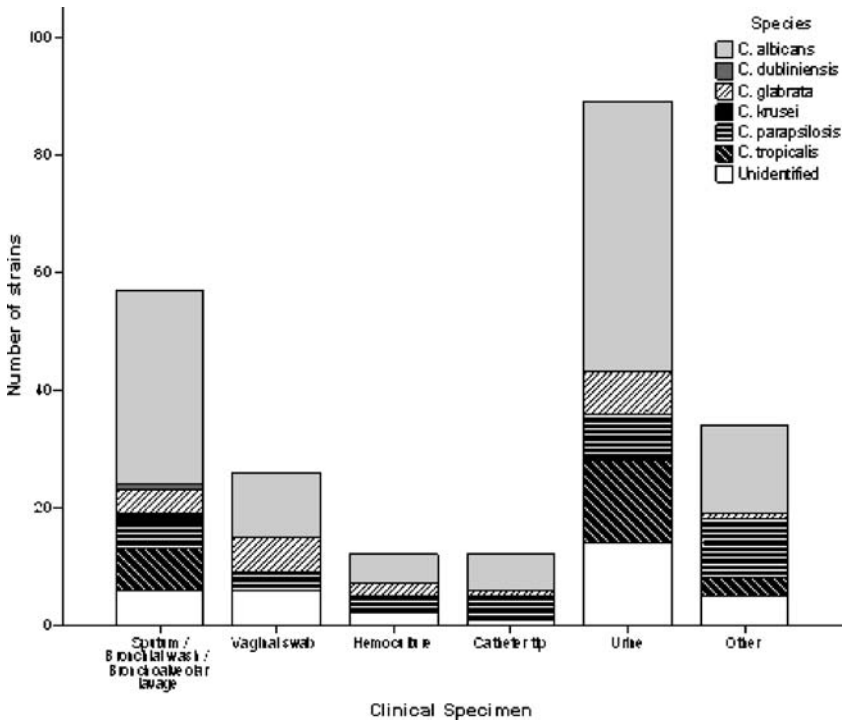

Fig. 1 Distribution of *Candida* species among clinical specimens

correctly identified the *C. dubliniensis* isolate. These results agree with previous studies (Marot-Leblond et al. 2006; Sahand et al. 2006) and indicate the accuracy of this test for discrimination between *C. dubliniensis* and *C. albicans*.

It should be noted that the levels of correct identification obtained in this study for the biochemical methods were lower than reported in previous studies (Loïez et al. 2006; Hata et al. 2007; Sheppard et al. 1998). This may be due, contrary to other studies, to the absence of supplementary tests, which are very time consuming and therefore disagree with the aim of this work that consisted in finding an expeditious methodology for *Candida* species identification.

In this work, we also compared the 24 and 48 h incubation times for Auxacolor and ID32C. Significant differences were found only for Auxacolor, showing better identification results for a 48 h incubation, which is in accordance with a previous study (Loïez et al. 2006).

In the present work, the prevalence and distribution of *Candida* species among clinical specimens was, for the first time, evaluated in HSM. The results obtained are in accordance with previous studies (Krcmery and Barnes 2002; Fleck et al. 2007), with *C. albicans* being the most prevalent species. However, NCAC levels are similar, corroborating the values in literature that denote the epidemiological shift of *Candida* pathogens perceived in the last years (Hazen 1995; Jarvis 1995).

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