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Rapid methods for identification of the most frequent clinical yeasts

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Over the past several decades there has been a significant increase in the number of fungal diseases. Although infections can occur in normal hosts, most of them are seen in patients who are immunosuppressed or immunocompromised such as those with AIDS or who have received transplants, corticoids or anticancer drugs [1]. Other predisposing factors responsible for yeast infections include overuse of broad-spectrum antibiotics, the presence of indwelling catheters, and intravenous drug abuse. In these opportunistic infections, due to microorganisms from endogenous or exogenous sources, an increasing diversity of opportunistic yeasts are implicated. The yeasts most commonly isolated from clinical specimens, in decreasing order of occurrence, include *Candida albicans, Candida glabrata, Candida tropicalis, Candida parapsilosis, Saccharomyces* spp, *Candida krusei, Candida guilliermondii, Rhodotorula* spp, *Trichosporon* spp and *Cryptococcus neoformans* [2]. However, *C. albicans* remains the most common species isolated, representing about 60% of all clinical isolates of yeasts [3].

In addition, opportunistic yeasts demonstrate various degrees of resistance *in vivo* and *in vitro* to common antifungal agents [4] and it is well-known that strains of *Candida lusitaniae* [5] on the one hand and *C. krusei* and *C. glabrata* [6-8] on the other are relatively resistant to amphotericin B and fluconazole, respectively. Consequently, mycoses are a growing medical problem requiring prompt diagnosis and early antifungal therapy.

There are a variety of methods available for identifying yeasts from clinical specimens. These include (i) conventional methods, e.g. the germ tube test, morphology studies, and carbohydrate utilization; (ii) rapid methods, e.g. enzymatic tests on colonies after primary isolation or directly on differential medium, latex agglutination tests and molecular biology based methods, and (iii) commercially available methods, e.g. manual biochemical panels (with carbohydrate assimilation and/ or fermentation and/ or enzyme profiles) and automated systems. This paper review the methods for rapid identification of yeasts, i.e., methods requiring less than five hours after isolation.

These rapid methods has been mainly developed for *Candida* species identification but several rapid tests can also be used for the identification of *C. neoformans*.

1. Germ tube test. The germ tube test (GT) [9], which is based on the fact that *C. albicans* produces short slender, tube-like structures when incubated at 35 to 37°C for 2 to 4 h in rabbit, sheep or foal serum, has been considered as the reference method for a long time. Although very reliable, the GT test is not without problems. Up to 5% of the strains of *C. albicans* may be germ tube negative and false-positive results can occur with other yeasts that produce germ tube-like structures, e.g. pseudohyphae [3,10,11]. These limitations, mainly due to the need for the preparation of the suspension and the microscopic examination by an experienced technician, have spawed research into developing suitable alternatives.

2. Enzymatic methods. Several investigators have demonstrated that *C. albicans* can be rapidly and accurately identified by using fluorogenic or/and chromogenic enzyme substrates [6,12-14]. These enzyme substrates can be tested (i) after the primary isolation of the organisms or (ii) they can be introduced into the primary isolation medium.

i) Methods carried out on colonies after isolation on a conventional medium. Many test kits (Table 1) have been commercialized, based on the colorimetric (fluorogenic or chromogenic) detection of the two enzymes L-proline aminopeptidase and ß-galactosaminidase.

Compared to the GT test, they have been shown to be more sensitive, more rapid (from 5 min to 2 h versus 2 to 4 h with the GT test) and more specific. Moreover, being easy to read, these tests do not require experienced technicians [2,6,10,15].

The detection of L-proline aminopeptidase in conjunction with the ß-galactosaminidase gives these tests a higher specificity than the chromogenic media which only detect the ß-galactosaminidase enzyme. Thus some *C. tropicalis* strains producing ß-galactosaminidase can be differentiated from *C. albicans* by negative detection of the L-proline aminopeptidase enzyme. Moreover, these tests are slightly more sensitive than the chromogenic medium. Performed on six *C. albicans* strains selected for their inability or poor ability to hydrolyse the respective chromogenic substrate on Albicans ID, Candiselect and/ or CHROMagar Candida plates, one of these tests, Murex CA, showed a higher sensitivity for the ß-galactosaminidase detection with four strains (Table 2, unpublished data).

The discrepant results between these different methods for ß-galactosaminidase detection might be due (i) to the nature of the substrate, (ii) the inoculum density or (iii) reaction conditions, notably pH conditions [13]. However, in contrast to the differential media, these enzymatic tests performed on the colonies require further handling and further incubation time, and do not facilitate recognition of mixed yeast population on primary plates.

ii) Direct identification on the primary differential media. Fluorogenic or chromogenic substrates for the ß-galactosaminidase enzyme have been introduced into the agar media according to a wide range of formula (Tables 3 and 4). These media have the advantages of allowing (i) identification of *Candida* species directly on the pri-

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Table 1. Commercially available enzymatic testing systems for rapid *C. albicans* identification.

Testing systems and References	Substrates used and time required	Capabilities[a]
Fluorogenic substrates for the ß-galactosaminidase detection: Wood lamp required		
1) ß-galactosaminidase and L proline aminopeptidase detections		
Albistrip (Lab M. Ltd, UK) R[c]: 6	4MU[b] N-acetyl galactosaminide Proline-p-nitroanilide 5 min at 37°C	S = 98% versus GT 98% Sp= 98% versus GT 95% UC = unknown
RapID Albicans (Biolife, Italy) R: 15	4MU N-acetyl galactosaminide + p-dimethylcinnamaldehyde 2 h at 37°C	S = 96.9% Sp = 100% UC = unknown
Albicans-Sure (Clinical Standards Laboratories, Inc., USA) R: 2	4MU N-acetyl galactosaminide p-dimethylcinnamaldehyde 5 min at room temperature	S = 99% Sp = 100% UC = $ 1
BactiCard Candida (Remel, USA) R: 2	4MU N-acetyl galactosaminide p-dimethylcinnamaldehyde 5 min at room temperature	S= 99.3% Versus GT 94.7% Sp = 99.6% UC = $ 1.76
2) ß-galactosaminidase detection only		
MUAG test (Biolife, Italy) R: 15, 19	4MU N-acetyl galactosaminide, directly on the colonies Wood Lamp required 3 min at room temperature	S = 99.6% to 97.6% Sp = 92.45% to 100% UC = $ 0.84
Chomogenic substrates for the two enzymes: no Wood lamp required		
ß-galactosaminidase and L-proline aminopeptidase detections		
Candi Albicans Screen (Carr-Scarborough Microbiologicals, Inc., USA) R: 11	p-nitrophenyl N ß acetyl ß-D galactosaminide p-dimethylcinnamaldehyde 90 min	S = 99% Sp= 99% UC = unknown
Murex C. albicans CA50 (Murex Diagnostics, USA) R: 2, 10	p-nitrophenyl N ß acetyl ß-D galactosaminide p-dimethylcinnamaldehyde 30 min at 37°C	S= 98.7% versus GT 95-97% Sp = 99.6% UC = $ 1.26

a : S = Sensitivity, Sp = Specificity, UC = Unitary Cost.
b : Methyl Umbelliferyl
c : R = References

mary isolation medium by means of the colony color, without further handling and (ii) an easier discrimination of *Candida* species colonies in mixed yeast populations.

The first two commercialized differential media Fluoroplate and SDCA-MUAG agar use fluorogenic substrates but the hydrolyzed fluorescent product must diffuse into the agar after 24 h of incubation. This is an inconvenient and enhances the difficulty in discriminating the different *Candida* species colonies, which may lead to an incorrect determination of the real number of associated species [15-18]. Furthermore, an UV light is required for the reading of the plates. For both these reasons chromogenic substrates are now preferably used. The performances of the different media regarding detection rate, sensitivity, specificity and bacterial selectivity are reported in tables 3 and 4.

All the media allow the identification of only *C. albicans* except CHROMagar Candida which allows the additonal identification of *C. tropicalis* and *C. krusei*. We would like to emphasize that according to other findings [25,29,31] and in contrast to the statements of Pfaller *et al.* [30] and Bernal *et al.* [26] our results confirmed that *C. glabrata* is not identifiable on this medium [20,21,28].

3. Immunological agglutination test. Recently, two latex agglutination tests have been commercialized by Fumouze Diagnostics (France) for the rapid identification of *C. albicans* and *C. krusei* colonies. The latex particules are coated with monoclonal antibodies that specifically react with a *C. albicans* antigen for Bichrolatex Albicans test and with a *C. krusei* antigen for Krusei color test. Bichrolatex Albicans test is made of red latex particles in suspension in a green dye and a positive reaction is traduced by red agglutinates on a green background. Krusei color test involves red-colored latex particles in suspension in an uncolored buffer: a positive reaction is revealed by the appearance of large red agglutinates.These methods have been shown rapid (five minutes), sensitive and specific [32-34].

Bichrolatex Albicans could be of special interest in cases of fungemia to identify *C. albicans* from blood culture bottles rapidly without the need for subculture on agar media [35]. Moreover this test showed a higher sensitivity for strains which gave false-negative or weakly positive reaction on several chromogenic media (Table 2) [32].

Table 2. Comparison of enzymatic identification on chromogenic media with that by latex agglutination and by Murex *C. albicans* test for negative or weakly positive enzymatic *C. albicans* strains.

	Result by test							
Strains[c]	**Albicans ID1**		**Candiselect**		**CHROMagar**		**Bichro latex albicans**	**Murex C. albicans**
	24h	48h	24h	48h	24h	48h		
G 6837	-	-	-	-	(+)[a]	+	+	NT[b]
G6848	-	-	-	-	(+)	+	+	-
D1356	-	-	-	-	-	-	+	-
G6841	-	-	-	-	(+)	+	+	+
8807094	-	(+)	-	(+)	(+)	+	+	+
8510011	(+)	+	(+)	+	(+)	+	+	+
361121	+	+	+	+	(+)	+	+	NT
313117	+	+	-	(+)	+	+	(+)	+

a : (+), weak
b : not tested
c : Strains isolated at the microbiological laboratory, Hôpital de l'Antiquaille

Table 3. Fluorogenic agar plates for the direct identification of *Candida* species.

Agar Plates and References	Enzyme detection	Capabilities[a]
	ß-galactosaminidase	
Fluoroplate Candida (Merck, Germany) R: 16-18	4MU[b] N-acetyl ß-D-galactosaminide Wood's lamp required Diffusion of the hydrolyzed fluorescent product after 24 h incubation	S = 46.9% at 24h, 93.8% at 48h Sp = 98.6% DR[c] = 90.4% BS : undetermined UC = $1.21
SDCA-MUAG agar (Biolife, Italy) R: 15, 19	4 MU-2-acetamide-2-desoxy -ß-D-galactosaminide + + Sabouraud agar Wood's lamp required Diffusion of the hydrolyzed fluorescent product after 24 h incubation	S = 98.4% to 99.4% at 48h Sp = 92.45% to 98.9% BS: undetermined UC = $1.54

a : S = Sensitivity, Sp = Specificity, DR = Detection rate, BS = Bacterial Selectivity, UC = Unitary Cost.
b : Methyl Umbelliferyl
c : In comparison, the detection rate of Sabouraud-chloramphenicol was 81.4%

Even with a perfect specificity, Krusei color could not be considered very useful, since for any non *C. albicans* yeast strain isolated from deep samples or from immunocompromised patients the sensitivity to antifungal agents must be determined to avoid resistance and in the meantime a broad-spectrum agent must be prescribed.

4. Biochemical and enzymatic panel.

i) Manual methods.

- Fongiscreen (Sanofi Diagnostics Pasteur, France) is a system allowing the identification of *C. albicans*, *C. tropicalis*, *C. glabrata* and *C. neoformans* after 4 h of incubation at 37°C. It includes seven tests: tetrazolium reduction, trehalose assimilation and five enzymes detection. A positive reaction is indicated by color change. Quindós *et al.* [36] reported sensitivity and specificity rates of 100%. Reliable, easy to perform and to read, this system remains relatively expensive ($ 4.54) [3,37].

- The RapID Yeast Plus System (Innovative Diagnostic Systems, USA) is a qualitative micromethod that uses conventional and chromogenic substrates for the identification of medically important yeasts within 4 to 5 h. Panels are inoculated with a heavy inoculum: visual turbidity approximately equal to a n° 3 Mc Farland turbidity standard. This inoculum of high density often requires 48 h of culture, which delays the identification, and may be a limiting factor to classify this method as a rapid one. After 4 to 5 h of incubation at 30°C, on the basis of the reactions, microcodes are constructed and used to search the manufacturer's computer service database for a species identification. Kitch *et al.* [38] and McNulty *et al.* [39] who identified 94.1% and 91% of the clinical yeast isolates, respectively, recommend this system for use in the routine laboratory for accurate same-day identification of clinically significant yeasts. But this system is very expensive ($ 8.99) and in our laboratory, with 154 clinical isolates, a rate of identification of only 85.3%, was obtained (unpublished data) due to difficulties in color appreciation notably with the five first cupules containing glucose, sucrose, raffinose, maltose and trehalose respectively and the three last ones containing substrates namely proline ß-naphthylamide, histidine ß-naphthylamide, and leucylglycyl ß-naphthylamide.

ii) Automated method

- The Baxter MicroScan system (Baxter MicroScan, Inc., USA) also provides yeasts identification within 4 h. The accuracy of this system is reported to be lower than those of the API 20C and Vitek systems. Land *et al.* [40], St Germain *et al.* [41] and Riddle *et al.* [42] reported rates of identification of 92%, 78% and 82% respectively.

Table 4. Chromogenic agar plates for direct identification of *Candida* species.

Agar Plates and References	Enzyme detection	Capabilities[a]
	1) ß-galactosaminidase only	
Albicans ID (bioMérieux, France) R: 3, 15, 16, 20-23	5 bromo 4 chloro 3 indolyl N-acetyl ß-D galactosaminide chromogenic substrate *C. albicans* = blue colonies	G: very small colonies for 20% of *C. glabrata* strains[b] S = 51.25% at 24h, 94.8% at 48h Sp = 99.6 % DR[c] = 86.6% BS : 0.83% (over 1447 samples tested) UC = $ 1.39
Candiselect (Sanofi Diagnostics Pasteur, France) R: 21, 23	Proprietary chromogenic substrate *C. albicans* = blue colonies	G: very small colonies for 5% of *C. glabrata* strains S = 31.8% at 24h, 95.15% at 48h Sp = 100% DRc = 84.3% BS: 1.1% (over 505 samples tested) UC = $ 1.39
	2) ß-D galactosaminidase and L-proline aminopeptidase	
Candichrom (International Microbio, France) R: 24	a tricompartimented plates with - a triphenyltetrazolium agar - a paranitrophenol N-acetyl ß-D galactosaminide agar - a cycloheximide + L-proline-paranitroanilide agar	G: similar to Sabouraud chloramphenicol agar except for *Geotrichum* (incubation at 37°C) S= 28,9% at 24h, 71,4% at 48h Sp = 100% BS = undetermined
	3) Several unspecified enzymes	
CHROMagar Candida (CHROMagar Society, France) R: 20, 21, 25-31	Proprietary mixture of chromogenic substrates *C. albicans* = green colonies *C. tropicalis* = blue colonies *C. krusei* = downy pink colonies	G: slightly smaller than on Sabouraud agar S = 8.8% at 24h, 89.1% at 48h Sp = 100% DRc = 88.5% BS : 3.24% (over 1143 samples tested) UC = $ 1.77

a : G = Growth, S = Sensitivity, Sp = Specificity, DR = Detection rate, BS = Bacterial Selectivity, UC = Unitary Cost.
b : an improved formula called Albicans ID2, giving larger *C. glabrata* colonies has been recently commercialized [23]
c : In comparison, the detection rate of Sabouraud-chloramphenicol was 81.4%

5. Other methods. Gas-liquid chromatography (MIS) in one day [43] cannot be recommended at this time for the routine identification of clinically isolated yeasts.

Molecular biology based methods using DNA probes, DNA fingerprints and amplification technics seem to be promising tools for the identification of *C. albicans* but extraction and purification of the target DNA still requires a lot of time [44]

Several rapid tests can also be used to identify *C. neoformans*. [3,45]

A direct microscopic examination of clinical material after India ink preparation may allow the detection of encapsulated *C. neoformans*. But this method, simple and inexpensive, suffers from a lack of sensitivity. Thus a latex test for detection of the cryptococcal capsular antigens is preferably done.

Three enzymatic tests namely urease, nitratase and phenoloxidase detections can provide a preliminary identification of *C. neoformans* in three to four hours.

- For the urease detection, a heavy inoculum of yeast is suspended in 0.5 ml of an urea-indol broth and incubated at 37°C. Urease positive organisms change the broth color from orange to purple in 2 to 4 h.

- Nitrate reductase is detected by sweeping a substrate-impregnated swab across several of the tested colonies to be tested and incubating the swab for ten minutes at 45°C. The swab is impregnated with 1% benzalkonium chloride as well as an excess of an inorganic nitrate and thus the complete reduction of the nitrate to amonia does not occur. After incubation, the swab is placed in a solution of sulfanilic acid and N, N-dimethyl-1-naphthylamine and observed for the development of a red color, which indicates the presence of nitrate reductase. *C. neoformans* does not use an inorganic nitrate substrate, while the others species in the genus may reduce nitrate.

- For the phenoloxidase detection, a plate of niger seed agar can be heavily inoculated and incubated at 37°C and a brown pigment will be produced by *C. neoformans* within four hours.

Ready-to-use panel: only Fongiscreen previously described allows the identification of *C. neoformans* within four hours.

Nowadays, identification of the most frequently encountered clinical yeasts is accessible to any microbiological laboratory. Indeed, the recent identification methods have diminished the importance of the morphological characters which require experienced staff, are subjectives and lack specificity and sensibility.

Enzymatic methods have been shown to be more rapid and more specific than the germ tube test, but they require the same handling and they do not facilitate discrimination of the different species on primary plates. All the chromogenic media have demonstrated higher detection rates than Sabouraud-chloramphenicol medium due, notably, to a better discrimination of species in mixed-

cultures and the possible identification of *C. albicans* without further handling.

In our microbiological laboratory, according to the patient population the hospital serves, the nature of specimens (sterile 49.45%, monofungal 71.66%, mixed-culture 28.33%), the recovery of yeasts (*C. tropicalis* 2.7%, *C. krusei* 2.39%), the cost-effectiveness of the medium, Albicans ID which gave the highest rate of *C. albicans* identification after 24 h incubation has been chosen. In addition, to compensate for the lack of rapidity of the chromogenic medium, in some specific clinical situations requiring early antifungal therapy, the agglutination test Bichrolatex Albicans is used.

For the other yeasts, the most important priority is to classify them as non-*C. albicans* due to their variable susceptibilities to antifungal agents, leading to the prescribing of a broad-spectrum antifungal agent instead of an azole, and secondly to identify them precisely. Thus a ready-to-use panel needing 24 to 48 h is used.

However, in the near future, the ideal reagent will be a chromogenic medium giving the fastest identification of most of the frequently isolated species including *C. glabrata* which is increasingly involved in pathology. At a later date a rapid system allowing determination of both identification and susceptibility to antifungal agents

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