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Phenotypic Characteristics of Yeasts of the Genus *Candida* and *Cryptococcus* in Differential Culture Media

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ABSTRACT

In the clinical mycology laboratory, the identification of yeast species is done by screening in specific media, such as chromogenic agar for *Candida* species and Niger seed agar for *Cryptococcus* species, both of which are of clinical interest. This study aimed to evaluate the growth and morphological characteristics of yeasts of the *Candida* and *Cryptococcus* species in different culture media. Yeast species included in the study were: *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. tropicalis*, *C. krusei*, *C. lipolytica*, *C. parapsilosis*, *C. metapsilosis*, *C. orthopsilosis*, *C. neoformans*, *C. gattii*, *C. flavescens*, and *C. albidus*. The media used were Sabouraud dextrose agar, Sabouraud dextrose broth, hypertonic Sabouraud broth (plus 6.5% NaCl), *Candida* chromogenic agar, methyl dopa agar, Niger seed agar and tobacco agar. Growth, color, size, presence of fringes, melanin and the appearance of the colony were evaluated. All isolates grew in the media used, except for the hypertonic Sabouraud broth; in *Candida* chromogenic agar, *C. albicans* and *C. dubliniensis* present a green color and *C. tropicalis* a blue color, while other species show colors including pink, purple, gray and white; in the Niger seed agar, *C. neoformans*, *C. gattii* and *C. flavescens* presented a brown color, while others had white colonies; in tobacco agar, the colors included white, cream and gray; and in methyl dopa agar, all colonies were white. Some isolates presented colonies with fringes in the tobacco, methyl dopa and Niger seed agar; the presence of melanin was observed by *Cryptococcus* isolates in the Niger seed and tobacco agar; the appearance of colonies in the media varied from opaque to shiny or mucoid, according to the isolate and the culture medium. All of the culture media used allowed the growth of the tested isolates, except for *C. lipolytica*, which did not grow in hypertonic Sabouraud broth. The isolates of *Cryptococcus*, *C. krusei* and *C. dubliniensis* presented a significant reduction of growth in hypertonic Sabouraud broth.

Keywords

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Introduction

The fungi of the genus *Cryptococcus* and *Candida* are among the most isolated in

clinical mycology laboratories. They are important fungi for humans and animals due to the diseases that they cause, such as primary or opportunistic mycoses (Kwon-

Chung and Bennett, 1992; Sidrim *et al.*, 2002).

Cryptococcus species are cosmopolitan and are found in the environment, and associated with bird excreta and different vegetable debris. They are agents of human and animal mycoses, with two main species being involved: *Cryptococcus neoformans* and *Cryptococcus gattii* (Kwon-Chung and Bennett, 1992). Some species of *Candida*, in turn, inhabit the body surface of animals and humans, but have also been isolated from the environment, including the air and water. *Candida albicans* is the best known species, accounting for the majority of cases of candidiasis (Lacaz *et al.*, 2002).

In the mycology laboratory, the identification of yeasts is performed by screening with classic tests, such as the germ-tube formation test, microculture in corn-tween 80 agar and auxanograms, which are generally more time-consuming. Other tests that are faster may be included, such as some miniaturized commercial tests that contain colorimetric, biochemical or enzymatic tests; these are more practical, but incur higher costs. The rapid confirmation of a fungal infection is important so that the treatment can be specific and started as soon as possible (Kwon-Chung and Bennett, 1992; Lacaz *et al.*, 2002).

The identification of a fungus from a clinical sample begins with the direct examination of the sample and followed by its isolation in culture. From the culture, the characteristics of the colonies, such as appearance and color, are observed in the specific media for fungal growth, for example Sabouraud agar, chromogenic agar, Niger seed agar and sunflower agar. Subsequently, a microscopic analysis of colonies of these cultures is performed, as well as additional biochemical tests, following the identification algorithm (Kwon-Chung and Bennett, 1992; Lacaz *et al.*, 2002).

Yeasts of the genus *Cryptococcus* are generally identified by the direct investigation of fungal structure in the clinical material, characterized by the presence of a capsule surrounding the cell, followed by culture. After the isolation of the colonies, the identification is performed as follows: evaluation of the *in vitro* production of urease, melanin production in media containing phenolic compounds, such as Niger seed agar, and confirmation of the presence of a capsule surrounding the cells. The differentiation between species of this genus is possible through physiological tests such as the assimilation of carbohydrates and nitrogen, from different sources. The species of *C. neoformans* and *C. gattii*, which are the most frequently isolated, can also be differentiated using bromothymol blue canavanine-glycine agar, in which *C. neoformans* is unable to grow, whereas *C. gattii* grow and change the media color from the original green to an intense blue (Freydiere *et al.*, 2001; Lacaz *et al.*, 2002; Pedroso *et al.*, 2007).

Species of the genus *Candida* are differentiated in the laboratory by phenotypic tests such as germ tube test, filamentation tests on corn agar plus tween 80, auxanograms and zymograms. The most frequent species in the clinical mycology laboratory are: *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. krusei* and *C. guilliermondii*. Another species, *C. dubliniensis*, although not very frequent, presents phenotypic and biochemical characteristics that are very similar to *C. albicans*, meaning that they need to be differentiated between for epidemiological purposes and also because they can present different responses to antifungals (Kirkpatrick *et al.*, 1998). A more intense green color in the chromogenic agar would be indicative of *C. dubliniensis*, while other practical tests, such as growth at variable temperatures, and morphology in tobacco agar, among others,

would be able to differentiate between these species (Freydiere and Guinet, 1997; Loreto *et al.*, 2010; Pasligh *et al.*, 2010). Currently, another species, *C. auris*, has emerged as a resistant species, sometimes presenting resistance to one or more classes of antifungals, which makes its identification and the *in vitro* susceptibility testing of antifungals extremely important (Arauz *et al.*, 2018; Arendrup and Patterson, 2017).

In the context of the use of culture media for screening and presumptive identification in mycology laboratories, this study aimed to evaluate the growth and morphocolonial characteristics of *Candida* and *Cryptococcus* species in different culture media.

Materials and Methods

Microorganisms and culture media

Twenty-one isolates of the *Candida* and *Cryptococcus* genera, including reference strains (INCQS, ATCC), controls (here called CFP, CP) and environmental (AMB 1) and clinical isolates (the others) used in laboratory, were evaluated. Specifically, the isolates studied were: three isolates of *Candida parapsilosis* (INCQS 40038, ATCC 22019 and CP 110), one of *Candida metapsilosis* (CP 111), one of *Candida orthopsilosis* (CP 112), two of *Candida albicans* (ATCC 90028 and P02), three of *Candida dubliniensis* (C142, P55V and P63), one of *Candida tropicalis* (P29R), two of *Candida krusei* (P61 and P115Rx), two of *Candida glabrata* (SV27 and SV113), one of *Candida lipolytica* (SV123), one isolate of *Cryptococcus flavescens* (AMB 1), one of *Cryptococcus albidus* var. *albidus* (INCQS 40190), one of *Cryptococcus gattii* (CFP 62), one of *Cryptococcus neoformans* var. *neoformans* (ATCC 28957) and one of *Cryptococcus neoformans* var. *grubii* (ATCC 90112).

Prior to the execution of the tests, samples stored in BHI-glycerol, kept at -20°C, were spiked onto Sabouraud agar and incubated at 30°C for 48–72 h; afterwards, two more seedings were performed on the same media for the complete reactivation of isolates and then the tests were performed.

The culture media tested were: Sabouraud dextrose agar (SDA), Niger seed agar (NSA), tobacco agar (TBCA), methyl dopa agar (MDA), chromogenic agar for *Candida* (CHRmA), Sabouraud dextrose broth (SDB) and hypertonic Sabouraud dextrose broth (HSDB, which is the SDB plus 6.5% NaCl).

In vitro tests

Suspensions of each isolate were made in sterile saline (NaCl 0.9%), with turbidity equivalent to 0.5 tube of the McFarland scale, prepared from a 48-hour culture on ASD.

From those suspensions, 10 microliters of each were seeded on the surface of agar contained in petri dishes (90x15mm). This was performed for each of the culture media (NSA, TBCA, MDA and CHRmA). Seeding was undertaken on four plates of each culture medium. Subsequently, the plates were incubated at 30°C for four days, with daily growth monitoring. The characteristics analyzed in all of the plaques were: growth, color and size of the colonies, formation of fringes in the periphery of the colonies and the production of melanin in the Niger seed, tobacco and methyl dopa agar.

Growth tests on SDB and HSDB were performed by transferring 20 µl of the suspension of each isolate in saline solution to two tubes containing SDB or HSDB (in duplicate). The tubes were incubated at 30°C for four days. SDB was used as a growth and turbidity control for comparison with HSDB. After incubation, the cultures were visually

examined for turbidity of the broth, which was compared to the McFarland scale tubes, and the test results were expressed according to turbidity.

Results and Discussion

All isolates of the genera *Candida* and *Cryptococcus* were grown on the media agar, methyldopa, tobacco and chromogenic *Candida* agar (Table 1).

In MDA, all colonies were white, except for *C. neoformans* and *C. gattii*, which were light brown. Regarding the size of the colonies, all colonies were small, except for *C. albidus* and *C. neoformans*, which had larger colonies when compared to those formed in SDA.

In the NSA, most of the isolates presented smaller colonies than in the SDA, with the exception of five isolates: *C. krusei*, *C. flavescens*, *C. albidus* and both *C. neoformans* isolates, whose colonies were similar in size to those observed in SDA. Fringe formation was observed in four isolates: *C. flavescens*, *C. gattii* and the two *C. neoformans*. Melanin production was observed in the colonies of *C. flavescens*, *C. gattii* and the two *C. neoformans* isolates.

In TBCA, all isolates had smaller colonies than those in SDA, ranging from bright or opaque to mucoid, and white, gray or beige to brown colors. Melanin was evidenced in two isolates of *C. neoformans* and one *C. gattii*. One isolate of *C. dubliniensis* and one of *C. krusei* were able to form fringes in TBCA.

In CHRmA, the colors of the colonies ranged from purple, green, beige, blue, white, and pink to gray; most of the isolates presented small colonies in comparison to the SDA and no isolates formed fringes. In addition, the colonies presented variable aspects, and all colonies of *C. glabrata* and *Cryptococcus*

spp. presented a brilliant appearance. In SDB, all isolates of *Candida* and *Cryptococcus* presented growth. In HSDB, the isolate of *C. lipolytica* did not show growth, while the *Cryptococcus flavescens* isolate also presented film formation on the broth surface (Table 2).

The detailed characteristics of the isolates tested are shown in Tables 1 and 2.

In this study, morphological characteristics of *Candida* and *Cryptococcus* species were evaluated in different culture media. These yeasts are clinically important because they are capable of causing serious diseases in humans, especially in immunocompromised individuals (Khawcharoenporn *et al.*, 2007; Pfeiller and Diekema, 2004).

The ideal culture medium for fungal growth and development is one that has all of the necessary substrates for the *in vitro* reproduction of microorganisms such as nitrogen, carbon, micronutrients, water, and others. For fungi, unlike bacteria, the isolation and identification of the main species can only be carried out with a few culture media in microbial laboratories (Lacaz *et al.*, 2002).

Some culture media are widely used, with different purposes such as: screening, selecting and differentiating specific isolates and the preliminary identification of isolates present in clinical samples; these include Sabouraud dextrose agar, corn agar, chromogenic agar, Niger seed agar, tobacco agar, methyldopa agar and hypertonic Sabouraud dextrose broth (Freydiere and Guinet, 1997; Loreto *et al.*, 2010; Menezes *et al.*, 2011; Pasligh *et al.*, 2010). Niger seed agar, for instance, is a selective and differential medium for the species *Cryptococcus neoformans* and *C. gattii*, which show colonies with brown pigment (Kwon-Chung and Bennett, 1992).

Table.1 Morphological characteristics of colonies of *Candida* spp. and *Cryptococcus* spp. on four different culture media

Species / isolate	Niger seed agar			Methyldopa agar			Tobacco agar			Chromogenic agar		
	Color	Size	Aspect	Color	Size	Aspect	Color	Size	Aspect	Color	Size	Aspect
<i>Candida parapsilosis</i> INCQS 40038	White	Tiny	Bright	White	Tiny	Bright	White	Tiny	Bright	Pink	Small	Bright
<i>C. parapsilosis</i> ATCC 22019	White	Tiny	Bright	White	Tiny	Bright	Beige	Small	Bright	Purple	Small	Opaque
<i>C. parapsilosis</i> CP 110	White	Tiny	Bright	White	Tiny	Bright	White	Tiny	Bright	Purple	Large	Opaque
<i>C. metapsilosis</i> CP 111	White	Tiny	Bright	White	Tiny	Opaque	White	Tiny	Opaque	Purple	Small	Bright
<i>C. orthopsilosis</i> CP 112	White	Small	Bright	White	Tiny	Bright	White	Tiny	Bright	Beige	Small	Bright
<i>C. albicans</i> ATCC 90028	White	Tiny	Opaque	White	Small	Bright	White	Tiny	Opaque	Light green	Small	Bright
<i>C. albicans</i> P02	White	Small	Bright	White	Small	Opaque	Beige	Tiny	Opaque	Light green	Small	Opaque
<i>C. dubliniensis</i> C142	White	Small	Bright	White	Small	Opaque	Beige	Tiny	Opaque	Green	Small	Opaque
<i>C. dubliniensis</i> P55V	White	Tiny	Opaque	White	Tiny	Opaque	Beige	Small	Bright	Green	Small	Bright
<i>C. dubliniensis</i> P63	White	Small	Opaque	White	Tiny	Opaque	Beige	Small	Opaque	Green	Small	Bright
<i>C. tropicalis</i> P29R	White	Small	Bright	White	Tiny	Opaque	White	Tiny	Bright	Blue	Large	Opaque
<i>C. krusei</i> P61	White	Small	Opaque	White	Tiny	Opaque	Grey	Tiny	Opaque	Beige	Large	Opaque
<i>C. krusei</i> P115Rx	White	Large	Bright	White	Small	Opaque	Beige	Tiny	Opaque	Purple	Large	Opaque
<i>C. glabrata</i> SV27	White	Small	Bright	White	Tiny	Bright	White	Tiny	Opaque	White	Small	Bright
<i>C. glabrata</i> SV113	White	Tiny	Bright	White	Small	Opaque	White	Tiny	Opaque	Purple	Small	Bright
<i>C. lipolytica</i> SV123	White	Small	Mucoid	White	Small	Opaque	White	Small	Opaque	Purple	Small	Opaque
<i>Cryptococcus flavescens</i> AMB 1	Light brown	Large	Mucoid	White	Small	Bright	Beige	Small	Bright	Pink	Small	Bright
<i>C. albidus</i> INCQS 40190	White	Large	Bright	White	Large	Bright	Beige	Small	Bright	Pink	Small	Bright
<i>C. gattii</i> CFP 62	Brown	Small	Mucoid	White	Small	Bright	Light brown	Tiny	Opaque	Pink	Small	Bright
<i>C. neoformans</i> ATCC 28957	Brown	Large	Mucoid	White	Small	Bright	Light brown	Tiny	Opaque	Grey	Small	Bright
<i>C. neoformans</i> ATCC 90112	Light brown	Large	Mucoid	White	Large	Bright	Brown	Small	Mucoid	Pink	Small	Bright

Table.2 Characteristics of the growth of *Candida* spp. and *Cryptococcus* spp. in hypertonic Sabouraud broth

Species	Growth in SDB*	Growth in HSDB*	Growth on SDB surface	Growth on HSDB surface
<i>Candida parapsilosis</i> INCQS 40038	3	3	0	0
<i>Candida parapsilosis</i> ATCC 22019	5	4.5	0	0
<i>Candida parapsilosis</i> CP 110	4	3	0	0
<i>Candida metapsilosis</i> CP 111	6	5	0	0
<i>Candida orthopsilosis</i> CP 112	5	5	0	0
<i>Candida albicans</i> ATCC 90028	5	4	0	0
<i>Candida albicans</i> P02	5	4	0	0
<i>Candida dubliniensis</i> C142	7	1	0	0
<i>Candida dubliniensis</i> P55V	7	0.5	0	0
<i>Candida dubliniensis</i> P63	7	0.5	0	0
<i>Candida tropicalis</i> P29R	6	5	0	0
<i>Candida krusei</i> P61	5	0.5	1	0
<i>Candida krusei</i> P115Rx	7	0.5	1	0
<i>Candida glabrata</i> SV27	8	6	0	0
<i>Candida glabrata</i> SV113	6.5	5.5	0	0
<i>Candida lipolytica</i> SV123	7	0	1	0
<i>Cryptococcus flavescens</i> AMB 1	3	0.5	1	1
<i>Cryptococcus albidus</i> var. <i>albidus</i> INCQS 40190	4	0.5	0	0
<i>Cryptococcus gattii</i> CFP 62	3	0.5	0	0
<i>Cryptococcus neoformans</i> var. <i>neoformans</i> ATCC 28957	1	0.5	0	0
<i>Cryptococcus neoformans</i> var. <i>grubii</i> ATCC 90112	4	0.5	0	0

Note: *: according to turbidity scale of McFarland; 0: Absent / negative test; SDB: Sabouraud Dextrose Broth; HSDB: hypertonic SDB

Tobacco agar is another medium used to verify the production of the melanin pigment in *Cryptococcus* species, but also for the differentiation between *Candida albicans* and *C. dubliniensis* (Loreto *et al.*, 2010; Silveira-Gomes *et al.*, 2011).

Chromogenic agar is a selective and differential medium for yeasts of the genus *Candida*, allowing differentiation between some species according to the coloration of the colonies; for example, the colonies of *C. albicans* and *C. dubliniensis* present a green color, while colonies of *C. tropicalis* show a blue color, colonies of *C. krusei* are lilac and dry, whereas other species may have white, cream or gray coloration (Odds and Bernaerts, 1994; Rousselle *et al.*, 1994).

In the present study, all isolates of *Cryptococcus* spp. grew in the NSA and TBCA. Regarding melanin expression, only the *Cryptococcus albidus* var. *albidus* was unable to produce this on TBCA; however, a brownish pigment was evidenced in NSA. The production of melanin in specific media by other species of *Cryptococcus* besides *C. neoformans* and *C. gattii* has been demonstrated in the literature (Menezes *et al.*, 2011; Pedroso *et al.*, 2007). None of the *Candida* species presented melanin in these media. The interesting thing in these findings was the production of melanin by *C. flavescens* in the NSA, a fact that has not yet been described in the literature, and which could be elucidated when the metabolic pathways related to the expression of laccase and phenol oxidase-like enzymes are studied and described.

MDA was proposed as a minimum, chemically controlled medium for the expression of melanin in *Cryptococcus* spp. (Menezes *et al.*, 2011). The melanin-producing species presented a very light brown color, which it is difficult to verify,

and is only possible when compared with an isolated control that does not express the pigment. On this culture medium it is interesting to note that pH seems to play a key role in the expression of melanin, since the pigment is evidenced at pH between 5.0-5.5. This medium only provided evidence of fringes for the species *C. tropicalis* and *C. krusei*, with no evidence of other characteristics that would be useful for the screening of other species in a routine laboratory.

The formation of fringes around colonies was observed in a few isolates in the media TBCA, MDA and NSA. This characteristic is presented by some isolates, but is dependent on the species and medium; therefore, it may not be a characteristic that can be used in the screening of microorganisms in the laboratory. However, it is a feature that can be used in studies which include morphotyping (Bacelo *et al.*, 2010).

TBCA was proposed as a form of differentiation between *C. albicans* and *C. dubliniensis*. According to the literature, colonies of *C. dubliniensis* in TBCA, as well as in NSA, have fringes due to the abundant mycelium and numerous chlamydo spores; this does not occur with colonies of *C. albicans*, which are smooth and do not produce chlamydo spores in those media (Liverio *et al.*, 2017; Loreto *et al.*, 2010). Contrary to the expected results, in the present study, however, only colonies of one of the three isolates of *C. dubliniensis* showed fringes, which suggests that this characteristic needs to be further explored and studied to determine the optimal incubation conditions and the interfering factors.

The development of *Candida* chromogenic agar was an evolution in relation to culture media, as it enabled the laboratories to identify *C. albicans*, the most frequent species

in clinical samples, with a high degree of certainty (Odds and Bernaerts, 1994). In general, in this study, the isolates showed different morphological aspects, ranging from bright to opaque. The results of this analysis are in agreement with the expected results and those which have been widely described in the literature. The differentiation between *C. albicans* and *C. dubliniensis* in chromogenic agar has been suggested by some authors (Liverio *et al.*, 2017); however, it seems to be difficult, and only when isolates from these two species are cultivated simultaneously is it possible to perceive the variation in the shade of green produced, according to the author's experience. On the other hand, different chromogenic culture media of different brands are on the market, and their performance is different, as shown by recent studies (Vecchione *et al.*, 2017). Therefore, it is up to each laboratory to evaluate the benefits and costs, and to adopt the method that best meets their needs.

The hypertonic Sabouraud dextrose broth was proposed to differentiate *C. albicans* from *C. dubliniensis*, as the latter shows inhibition or an expressive reduction of growth (Mahelová and Ruzicka, 2017; Silveira-Gomes *et al.*, 2011). In the present study, it was observed that many isolates had a decrease in growth in HSDB, compared to SDB, according to the turbidity scale. However, according to the literature, *C. dubliniensis* presented a decrease when compared to the McFarland turbidity scale; the three isolates of *C. dubliniensis* presented turbidity equivalent to tube 7 in the SDB, while the turbidity in HSDB ranged from 0.5 to 1. In HSDB, isolates of *C. krusei* and *C. lipolytica* showed no growth or presented significant growth reduction. *Cryptococcus* isolates showed variable growth in SDB, and decreased growth in HSDB. As the isolates of *Cryptococcus* are aerobic, it is expected that there is little growth in static incubation in liquid medium,

but it was possible to verify a reduction of growth in the hypertonic broth. *Cryptococcus* species require the presence of oxygen for growth, so they will have difficulty growing in liquid media (Kwon-Chung and Bennett, 1992). This is because the culture medium is a suspension of microorganisms; when inoculated, the cells tend to go to the bottom of the tube, so the absence of oxygen will reduce or impede growth. Incubation of the culture media in this study was performed in a static manner; however, if the incubator is shaken, this interference can be eliminated.

Also, it was observed that the isolates of *Candida krusei*, *Candida lipolytica* and *Cryptococcus flavescens* showed a growth film on the surface of the broth in SDB. This has not been previously described, and may help the laboratory in identify those species. In general, all of the media allowed the growth of the tested isolates, with the exception of *C. lipolytica*, which did not grow in HSDB. On the other hand, the isolates of *Cryptococcus*, *C. krusei* and *C. dubliniensis* showed a significant reduction of growth in HSDB.

Phenotypic methods remain the main way to identify or screen fungi that are isolated in the medical laboratory, especially in small laboratories, with a small sample processing capacity, and which are located far from major centers. More advanced methodologies such as those based on DNA or proteomics are restricted to large laboratories and/or reference centers because of the high cost and complexity of the techniques. Thus, studies that seek to improve the screening of species in culture media already used in the routine laboratory, as well as the testing of other media should be encouraged and disseminated, so that the most frequent fungal species in mycology laboratories can be identified with greater accuracy.

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