A new caffeic acid minimal synthetic medium for the rapid identification of *Cryptococcus neoformans* isolates

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Summary Melanin production is one of the most important criteria for rapid identification of *Cryptococcus neoformans*. Most of the media described in the literature for identifying *C. neoformans* are very complex; they contain many organic or inorganic compounds and are difficult to prepare and store. The new minimal synthetic caffeic acid medium described in this paper is simpler to prepare, convenient and constitutes an interesting new medium for the rapid identification of *C. neoformans* isolates.

Key words *Cryptococcus neoformans*, Caffeic acid medium, Phenoloxidase, Identification

Un nuevo medio sintético mínimo para la identificación rápida de aislamientos de *Cryptococcus neoformans*

Resumen La producción de melanina es uno de los criterios más importantes para la identificación rápida de *Cryptococcus neoformans*. La mayoría de los medios de cultivo descritos para la identificación de *C. neoformans* son muy complejos: contienen muchos compuestos orgánicos e inorgánicos y son difíciles de preparar y almacenar. El nuevo medio mínimo sintético con ácido caféico, descrito en este trabajo, es más sencillo de preparar y constituye un nuevo medio apropiado para la identificación rápida de *C. neoformans*.

Palabras clave *Cryptococcus neoformans*, Medio con ácido caféico, Fenoloxidasa, Identificación

Mycoses are a growing medical problem requiring a prompt diagnosis to facilitate an early antifungal treatment. *Cryptococcus neoformans* has risen to a worldwide highly recognizable major opportunistic pathogen with deadly consequences in immunocompromised patients, particularly in those with AIDS. Cryptococcosis, in the pre-high activity anti-retroviral therapy era, became a major cause of disease and death among patients with AIDS and was the commonest life-threatening mycosis in these patients. Drug users, homosexual men, polytransfunded patients, hemophiliacs, and immunocompromised hosts, among others, are at high risk of cryptococcosis [3,9,15].

Melanin production by phenoloxidase activity is a distinctive and characteristic property of *C. neoformans* isolates [2,4,6]. The ability to produce these melanin pigments is one of the most used criteria for the identification of *C. neoformans* clinical and environmental isolates and for the evaluation of *Cryptococcus* virulence [13,17]. Melanin production is usually tested in a proper agar medium containing a precursor of melanin. For this purpose, agar media containing DOPA, caffeic acid, birdseed or sunflower extracts have been reported so far [1,5-8,10-12,14,16,18,19]. Vidotto *et al*. [20] have developed a vitamin-free minimal synthetic medium for *C. neoformans*. In the present paper, we report the comparison of melanin production by clinical and environmental isolates of *C. neoformans* and other medically important yeasts on a new minimal synthetic caffeic acid medium (MSCAM).

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Materials and methods

The original caffeic acid agar medium (CAAM) –composition per liter: glucose 5 g; ammonium sulphate 5 g; yeast extract 2 g; potassium phosphate 0.8 g; magnesium sulphate 0.7 g; caffeic acid 0.18 g; noble agar 20 g; ferric citrate solution 4.0 ml (2 mM)– was modified. The new medium, minimal synthetic caffeic acid medium (MSCAM), only contains caffeic acid and ferric citrate (caffeic acid 0.18 g/l; noble agar 20 g/l and 4 ml/l of ferric citrate solution).The medium was sterilized in an autoclave at 120 °C for 15 min. Twenty milliliters of the medium were dispensed in Petri plates under sterile conditions.

The following clinical and environmental isolates were studied: 170 human isolates of *C. neoformans* from the Adolfo Lutz Institute (Sao Paulo, Brazil) and from the Sezione Malattie Infettive (University of Torino, Torino, Italy), 10 *C. neoformans* isolates from bird droppings from the University of Messina (Messina, Italy), 10 *Candida albicans* strains from the Sezione Malattie Infettive (University of Torino), 10 *Candida dubliniensis* strains from the Medical Mycology Laboratory (Universidad del País Vasco, Bilbao, Spain), two *Candida glabrata* isolates, and one isolate each of *Candida tropicalis* and *Saccharomyces cerevisiae* from the Sezione Malattie Infettive (University of Torino). Identification of isolates was confirmed by conventional mycological methods such as colony and microscopic morphology, urease production, nitrate reduction, germ tube test, chlamydoconidium production on corn meal agar, and carbohydrate assimilation patterns using ATB ID 32 C kit (bioMérieux, France).

The isolates and strains were transferred onto fresh malt agar slants and incubated at 25 °C. After five days a loopful of the colonies was inoculated on MSCAM, and incubated at 37 °C in the dark for 4 h, and then for 24 h. Sterile normal CAAM was used as control. Each isolate was tested three times in triplicate. Depending on the brown pigment produced by each of the *C. neoformans* isolates tested after 4 h and 24 h of incubation at 37 °C, five groups were created: without pigment production (0), very poor pigment production (1), poor pigment production (2), good pigment production (3) and very good pigment production (4). The data obtained from the experiments performed three times in triplicate were evaluated by using the statistical Sign test.

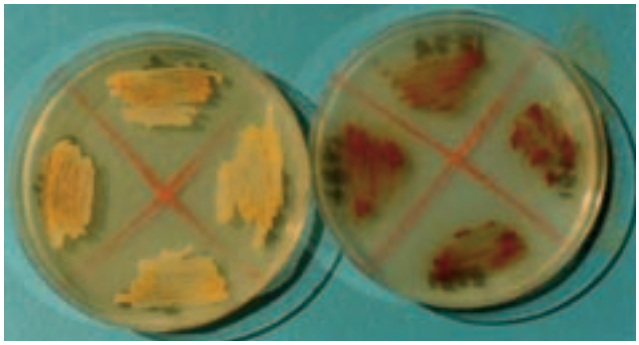

Figure. Different pigment production by a *C. neoformans* isolate from an AIDS patient after 24 h incubation at 37 °C on the CAAM (left) and on the new MSCAM (right).

Results

All *C. neoformans* isolates produced brown pigment after 4 h and 24 h of incubation at 37 °C. After 4 h, 132 (73 %) *C. neoformans* isolates showed a poor pigmentation (categories 1 and 2) in the new MSCAM and 48 (27%) isolates showed highly-pigmented colonies (categories 3 and 4). At the same incubation time (4 h) using CAAM, 160 (89%) *C. neoformans* isolates showed a poor pigmentation of the colonies (categories 1 and 2) and 20 (11%) a notable pigmentation (categories 3 and 4) (Table). After 24 h of incubation, colonies from 146 (81 %) strains showed a rich pigmentation (categories 3-4) in the new MSCAM medium and, on the contrary, only colonies from 85 (47 %) *C. neoformans* strains showed a rich pigmentation (categories 3 and 4) in CAAM (Table).

In the new MSCAM after 4 h at 37 °C, 170 *C. neoformans* isolates showed higher pigment production (1.9 mean) than the same isolates tested in the normal CAAM (1.7 mean).The Sign test showed significant differences between both media ($p = 0.006$). It is interesting to outline that *C. neoformans* isolates were able to produce a low pigmentation in the new MSCAM after 2 h at 37 °C. On the contrary no pigmentation was observed in the normal CAAM (data not shown). After 24 h, *C. neoformans* isolates in the MSCAM produced higher pigment production (3.2 mean) than the same strains tested in the normal CAAM (2.5 mean) (Figure). The Sign test resulted very significant ($p = 0.0001$). No pigmentation was observed at 4 h and 24 h in any of the tested isolates from the species *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. tropicalis* and *S. cerevisiae*.

Table. Pigment production values of 180 *Cryptococcus neoformans* strains tested on MSCAM and CAAM after 4 h and 24 h incubation at 37 °C.

		Incubation time			
		4 h		24 h	
Medium		1 and 2*	3 and 4*	1 and 2	3 and 4
MSCAM	No. of isolates (%)	132 (73)	48 (27)	34 (19)	146 (81)
CAAM	No. of isolates (%)	160 (68)	20 (11)	95 (53)	85 (47)

*Pigmentation category: 1 and 2 = very poor and poor colony pigmentation; 3 and 4 = strong and very strong colony pigmentation.

Discussion

The only two compounds contained in the MSCAM allowed more rapid growth and pigmentation of colonies from *C. neoformans* isolates than the most common used CAAM. This new medium, MSCAM, is easy to prepare, store and costs less than CAAM. Absence of glucose in the new medium increases pigment production in *C. neoformans* clinical and environmental isolates. According to our results the absence of glucose does not interfere in *C. neoformans* phenoloxidase activity. It is also possible to hypothesize that bivalent or monovalent ions such as Ca^{++}, NH_4^{++} or K^+ do not interfere in *C. neoformans* phenoloxidase activity. According to Polacheck [16] reduction of ammonium sulphate does not interfere with melanin synthesis and consequently with *C. neoformans* pigmentation.

The concept of using differential media for isolating specific fungal pathogens is not new but problems encountered with differential media used for identification of *C. neoformans* have included a elevated cost, a complex medium preparation, and ill-defined interpretation. MSCAM is inexpensive and easy to prepare and store; moreover, it allows a rapid and clear identification of *C. neoformans* isolates from human and environmental sources.

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