

Identification of *Cryptococcus neoformans* isolates using Staib agar without creatinine

Identificación de Aislados de Cryptococcus neoformans Usando Agar Staib sin Creatinina

**Nardelli, Vanessa¹; Pérez, Celina¹;
Mata-Essayag, Sofía¹; Colella, María Teresa¹;
Roselló, Arantza¹; Hartung de Capriles,
Claudia¹; Landaeta, María Eugenia²;
Olaizola, Carolina¹ y Magaldi, Sylvia¹**

¹ Sección de Micología Médica, Instituto de Medicina Tropical, UCV, Caracas. ²Cátedra de Microbiología, Escuela de Medicina Luis Razetti, Universidad Central de Venezuela. Caracas, Venezuela.
E-mail: somae50@hotmail.com

Resumen

En micología, el cultivo es la mejor manera de realizar el diagnóstico definitivo de una micosis. Muchos tipos de medios han sido desarrollados y modificados para tal fin, utilizando las propiedades bioquímicas de los hongos para reconocerlos. Los medios más comunes son Sabouraud, agar Malta, agar maíz, agar Papa-dextrosa, y agar Staib. Este último ha sido ampliamente usado para la identificación de levaduras del género *Cryptococcus* y en otros hongos. El objetivo de este estudio fue demostrar la utilidad del agar Staib y de un medio de Staib modificado, sin creatinina, en el aislamiento e identificación de hongos patógenos. Cuarenta y seis aislados de *Cryptococcus neoformans* más un control fueron identificados por criterios morfológicos y bioquímicos, usando la prueba de ureasa (agar urea de Christensen) y cultivo en medio Sablac. Las cepas fueron transferidas a placas de agar Staib estandar y de agar Staib sin creatinina. Las placas fueron incubadas a temperatura ambiente (26-28°C) durante una semana y luego fueron evaluadas las características morfológicas de las colonias y producción de fenoloxidasa. En 43 (91.5%) de las cepas estudiadas, se observó producción de fenoloxidasa (presencia de colonias marrones) tanto en el agar estandar como en el agar sin creatinina. Conclusión: el agar Staib sin creatinina es un medio excelente para la identificación de *C. neoformans*, con las ventajas de un bajo costo y fácil preparación.

Palabras clave: Agar Staib, *Guizotia abyssinica*, *Cryptococcus neoformans*, creatinina.

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Abstract

In mycology, culture is the best way to demonstrate and identify pathogenic fungi. Many kinds of media have been developed and modified, using fungal biochemical properties to recognize them. The most common media are Sabouraud, Malt Agar, Cornmeal agar, Potato-dextrose agar, and Staib agar. Staib agar has been widely used for identification of yeasts from the genus *Cryptococcus* and other fungi. The aim of this study was to demonstrate the usefulness of Staib agar, and of a modified Staib medium without creatinine, in the isolation and identification of fungi. Forty-six *Cryptococcus neoformans* strains plus one control strain were identified by morphological and biochemical criteria, using urease test (Christensen urea agar) and culture in Sablac medium. The strains were then transferred into standard Staib and Staib without creatinine agar plates. Morphological features and phenoloxidase production were evaluated after one week incubation at room temperature (26-28°C). Culture in standard and modified Staib agar yielded brown color colonies (phenoloxidase production) in both media in 43 (91.5%) cases. Staib agar without creatinine is an excellent medium for identification of *C. neoformans*, with the advantage of a low cost and easy preparation.

Key words: Staib agar, *Guizotia abyssinica*, *Cryptococcus neoformans*, creatinine.

Introduction

Staib agar medium has been widely used for isolation and identification of yeasts from the genus *Cryptococcus*. It was developed in 1962, by the German mycologist Friedrich Staib, who thought of a culture medium made of *Guizotia abyssinica*, an oval black seed used for bird feeding. It is also used in India and Ethiopia for oil production (1, 2).

In his first studies, Staib employed powdered canary droppings, which is used by *Cryptococcus neoformans* as a nitrogen source. Thus, Staib observed a color change in the colonies, which grew in this medium, turning into a brown color after 4 days of incubation (3).

Later, Staib found that the color variation was not only dependent on the canary droppings, but on the food these birds consumed, principally made of *Guizotia abyssinica* seeds (3).

These seeds contain caffeinic acid, among other phenolic compounds, in which O-diphenol undergoes an oxidation process by action of the phenoloxidase enzyme, pro-

duced by *C. neoformans*, originating a melanin pigment which covers the yeast wall, thus turning the colonies growing in culture to a dark brown color. This feature differentiates *C. neoformans* from other yeasts. This reaction was named "Brown color effect" (BCE) by this author (2, 4). Then, BCE depends on the enzyme diphenoloxidase, an antioxidant, which helps the yeast to survive inside the host by the production of melanin (5).

This medium is prepared with glucose, creatinine and potassium phosphate (6). Creatinine is assimilated by *C. neoformans*; this is possible only in the presence of thiamine or other compounds like thiazol and pirimidine, which are in the seeds. Creatinine is used by the yeast as nitrogen source, its assimilation is especially important in serotypes B and C, but not A and D. This assimilation has been demonstrated by the presence of a green diffusible pigment (1, 2, 3, 7).

Staib medium is used routinely since the beginning of the AIDS epidemic, which increased the frequency of opportunistic infections, including fungal. Culture of samples (especially sputum) from AIDS patients di-

rectly onto this medium shows brown colonies of *C. neoformans* (5, 7-13).

For these reasons, we started to use this medium in our medical mycology laboratory, for the isolation and identification of *C. neoformans* in clinical samples from AIDS patients.

Initially, Staib agar was being prepared with the traditional method, but further on in the study there was a failure in the supply of some of the compounds, especially creatinine. For this reason the medium was modified, and prepared without creatinine. Surprisingly, *C. neoformans* colonies showed a deeper brown color than in the original Staib agar. We decided to continue using this medium with excellent results.

The aim of this study was to assess the usefulness of Staib agar without creatinine for the identification of *C. neoformans*, by the observation of the presence of pigment, macro and microscopic morphological development and time of growth, using strains from the Medical Mycology Department Culture Collection.

Materials and Methods

After certifying their pureness and viability, forty-six *C. neoformans* strains from the Medical Mycology Department Culture Collection were identified by morphological and biochemical criteria, using urease test (Christensen urea agar) (14) and culture in Sablac medium, which allows better capsule development. They were also identified with the Microscan™ Dade Behring Automated System.

C. neoformans (WC 1400 IMT) and *Candida albicans* (ATCC 90028) were used as urease positive and negative control isolates, respectively.

Additionally 5 isolates of *Candida dubliniensis* (S2-5, S2-6, S2-14, S636 and S645)

from the Health Science Center, University of Texas (supplied by Dr. Anette Fothergill) were used to assess their development in Staib agar with and without creatinine.

All strains were then transferred into standard Staib and Staib without creatinine agar plates (two isolates per plate), they were incubated at room temperature (26-28°C) for one week, and then the morphological features were evaluated.

Canavanine-glycine-bromotimol blue (CGB) medium (15) was used to identify *Cryptococcus* isolates. All isolates were inoculated in this medium and incubated at room temperature for 5 days. Interpretation of results: yellow color: *C. neoformans var neoformans*, dark blue color: *C. neoformans var gattii*.

Descriptive statistics were used to analyze data obtained, using frequency tables, and percentage and sensitivity determination.

Results

During the course of this study we were forced to use Staib medium without one of its main components, creatinine. The experiments were continued using the modified medium. Later in the study, we were able to find the creatinine and nevertheless decided to use both kinds of Staib medium, the original and the modified.

Forty-seven *C. neoformans* strains used for the study were tested for urease production, being all positive. The same strains were tested for phenoloxidase production, culturing in standard and modified Staib agar. They yielded brown color colonies in both media in 43 (91.5%) cases (Figura 1, 2). In 4 (8.5%) of the studied strains, the colonies remained cream color, in spite of being urease positive and identified by automated Microscan™ method (Table 1).

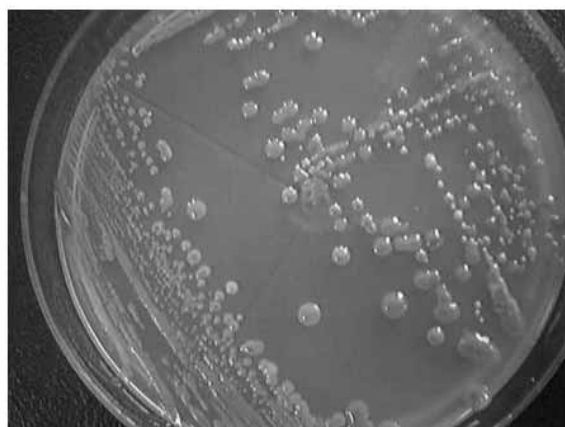


Figura 1. *Cryptococcus neoformans* in standard Staib agar.

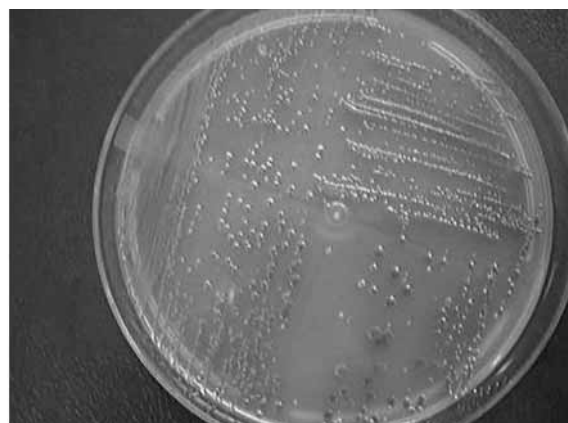


Figura 2. *Cryptococcus neoformans* in Staib agar without creatinine.

Table 1. Phenotypic differences of *Cryptococcus neoformans* in Staib agar with and without creatinine.

Media	“Diffusible Pigment”	Colony Color	Strains	Microscopic Features	Growth Time (Hours)	Brown color colonies (Days)	Growth Temperature (°C)
Standard Staib	No	Light brown	33	Encapsulated yeasts	48-72	5-8	26-28
		Cream	–	–	–	–	–
		Light brown	10	Encapsulated yeasts	24-48	5-8	26-28
	Green	Cream	4	Encapsulated yeasts	24-48	–	26-28
Staib without Creatinine	No	Dark brown	43	Encapsulated yeasts	48-72	5-8	26-28
		Cream	4	Encapsulated yeasts	48-72	–	26-28

From the 47 isolates inoculated in CGB medium, only one showed dark blue color, which suggested *C. neoformans var. gattii*. This isolate came from a HIV seronegative patient. In standard Staib agar, colonies were light brown color, without green diffusible pigment; in Staib without creatinine, colonies were dark brown.

Five *C. dubliniensis* strains were inoculated on standard Staib agar and Staib agar without creatinine. In standard Staib agar, all 5 isolates showed the same characteristics:

rough colonies, and formation of chlamydoconidia. On the other hand, on Staib agar without creatinine, neither of the isolates showed these features. Instead, colonies were smooth, and no chlamydoconidia were formed.

Discussion

In mycology, culture is the best method used to demonstrate the presence of pathogenic fungi. This is obtained by culturing a

clinical sample in a nutrient rich medium, which “feeds” the fungus, in favorable temperature and time conditions, until it can be seen and identified.

During the history of mycology dozens of culture media have been developed, which have been made to highlight the morphological properties of different pathogenic fungi. The most common are Sabouraud agar, Malt agar, Cornmeal agar, Potato-dextrose agar, and Staib agar (6, 16, 17).

Staib in 1962, developed Staib agar, using *Guizotia abyssinica* seeds (1). This medium has been very useful in the identification of *Cryptococcus* strains, and moreover since the beginning of the AIDS epidemic, which has provoked a rise in fungal opportunistic infections.

Forty-three (91.5%) out of 47 *C. neoformans* strains, showed light brown colonies on the standard Staib medium and dark brown colonies on the modified Staib medium (without creatinine). There is no explanation for the different brown colors observed.

The other 4 (8.5%) strains showed cream color colonies on both media. Being these isolates from a culture collection, there is no information about their original color. Since these strains were 20 years old, and conserved in Castellani medium (distilled water)¹⁸, maybe they could have lost the capacity of production of phenoloxidase in time, which is necessary to produce melanin (2, 5, 19-22). Melanin production is considered one of the main pathogenic factors of *C. neoformans* (22).

On the other hand, Staib, in 1962 determined that serotypes B and C, but not A and D assimilate creatinine from the medium, evidenced by the presence of a green diffusible pigment (1). In our study we found 14 strains that produced green diffusible pig-

ment. We initially presumed that these were serotype B and C strains. However, when the isolates were inoculated in CGB medium, only one showed the characteristically dark blue color of *C. neoformans var gattii*. CGB medium is very specific for identification of this variety (15), and it is also useful for epidemiological studies of Cryptococcal disease.

Bennett et al. (1978) also found that certain *C. neoformans var gattii* (serotypes B and C) produced a green diffusible pigment in Staib medium. This phenomenon did not occur with *C. neoformans var neoformans* (serotypes A and D), which have a very slow assimilation of this compound. They explained that when serotypes B and C assimilate creatinine, it is metabolized producing ammonium, turning the medium to an alkaline pH and green color (7, 23, 24). For this reason the term “diffusible green pigment” used in published papers, actually refers to a color change in the medium provoked by the pH variation, and not to production of pigment. Since we didn’t find similar results, we concluded that Staib medium is not a sensitive or specific way of identifying B and C serotypes.

Bennett et al in 1978, reported that not all *C. neoformans* strains assimilate creatinine in the same way. *C. neoformans* Serotype A grew more slowly (48-72 hours) in this medium (7), which also made creatinine assimilation slower. In our study, strains that assimilated creatinine between 48 and 72 hours of observation could correspond to serotype A (Table 1). This will be verified in future studies of strain serotyping.

In our revision of international literature, we found that in 1978, Paliwal and Randhawa (21) prepared a simplified *Guizotia abyssinica* seed medium, eliminating glucose, creatinine, and phosphate. This was evaluated for the isolation and presumptive

identification of *C. neoformans*. The results of this study demonstrated that the simplified or modified *G. abyssinica* seed agar was superior to the complete medium for the rapid development of the brown pigment by *C. neoformans*. This finding is very similar to our results.

In the case of *C. dubliniensis*, Staib agar without creatinine was not useful in identifying these strains, as distinctive features were not present in this medium (rough colonies, presence of chlamydoconidia). For this reason, standard Staib agar continues to be the best medium to identify *C. dubliniensis*, as well as differentiating it from *C. albicans* (28).

Therefore Staib agar without creatinine can be used as a cheaper option, for the identification of *C. neoformans*, especially in laboratories with restricted economic resources.

It can be used for culture of clinical samples from patients with or without immune suppression, or AIDS patients, when cryptococcal disease is suspected, as recommended by other authors in the literature (5, 8, 10-12, 25, 26, 27, 29-31).

This medium was not useful in the morphological identification of *C. dubliniensis* or recognition of *C. neoformans* serotypes.

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