ARTICLE

GENOTYPES OF CRYPTOCOCCUS SPP ITS BEHAVIOR AS BIOFILM

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ABSTRACT

Eight genotypes are recognized in the Cryptococcus neoformans / Cryptococcus gattii complex : VNI and VNII (C. neoformans var. grubii), VNIII (C. neoformans hybrid AD), VNIV (C. neoformans var. neoformans) and VGI genotypes , VGII, VGIII and VGIV of the species C. gattii . The objective of the present work was study the capacity of the different genotypes for the formation of biofilms (BP) and evaluate staining techniques for its demonstration. For this, genotyping was carried out : it was carried out by means of PCR-RFLP. With each strain, the dynamics of BP formation in vitro was studied , with a glassware (DV) as an abiotic support in Sabouraud broth.

They were extracted after 24, 48, 72 hours, and in some, after a week. Two colors were used: nigrosine and crystal violet, they were observed with an optical microscope and the score used by us was applied. The results were 4, 3 and 1 at 24 hours; from 5, 3 and 4 at 48 hours and from 4, 3 and 4 at 72 hours for VNI, VNII and VNII respectively.

Scoring: 1, 2 and 1 at 24 hours; 2, 2 and 1 at 48 hours and 1.1 and 1 at 72 hours for the C. gatti complex , VGI, VGII and VGIII respectively. One week later, the score was 5 for NIV and 3 for VGI. Thus, the following conclusions were reached: the VNI genotype was the one that formed the most vigorous BP and its presence in meningitis, especially in people living with HIV could explain some treatment failures.

KEY WORDS : Cryptococcus spp Biofilm Score

INTRODUCTION:

Cryptococcosis is an opportunistic mycosis with worldwide distribution, whose first clinical descriptions date from 1894-1895 [1].

The disease is caused by yeasts of the genus Cryptococcus: Cryptococcus neoformans / Cryptococcus gattii complex.

Within this complex five serotypes (A, B, C, D, and the AD hybrid) and eight genotypes are recognized: VNI and VNII (C. neoformans var. grubii), VNIII (C. neoformans hybrid AD), VNIV (C. neoformans var. neoformans) within C. neoformans and genotypes VGI, VGII, VGIII and VGIV that correspond to the species C. gattii (Table 1) [2,3] .

The immunosuppressed population is at greater risk of suffering from the disease: HIV-positive patients, splenectomized patients, patients with lymphoproliferative diseases, corticosteroid therapy, transplant recipients, and/or malnourished patients [4,5] .

These capsulated yeasts enter the body through inhalation, which triggers a primary pulmonary infection that, if not eradicated, can spread to the viscera, skin and/or CNS, which is its main target.

It can also generate a subclinical and/or latent infection that is reactivated years later in case of immunosuppression due to a true autoinfection [6,7,8] .

Other documented routes of contagion are by contiguity -cutaneous cryptococcosis- and oral -digestive cryptococcosis- [9, 10] .

C. neoformans is distributed globally, its main reservoir

being bird guano, while C. gatti is found in tropical and subtropical climates, and is associated with different trees such as Eucalyptus [11]. (Table 1).

Cryptococcus spp, like most microorganisms, is capable of forming BP, its main mode of presentation in the environment (80%) and in infections (65%) [12, 13, 14].

This is considered an important virulence factor, favors genetic exchange, antifungal resistance and immune evasion. They also constitute a survival factor in the environment as well as in humans [15,16].

Cryptococcosis can be diagnosed by different methods such as negative staining or India ink staining, which stains the entire preparation except the capsule and allows a presumptive diagnosis of cryptococcosis to be made.

The sensitivity of the stain ranges from 25-50% in cases of meningitis, although in patients with AIDS it may be higher. Giemsa (GC) staining is also used.

There are other stains used in histopathology, such as methenamine-silver or periodic acid Schiff (PAS), which allow identification by size and narrow-based budding.

The cultivation and identification that is carried out by the conventional methods of assimilation and fermentation of sugars, require up to 14 days of incubation.

Alternatively, commercial automatic or semi-automatic systems can be used. The detection of capsular antigen by a latex technique, which is useful in serum, CSF, urine, and even respiratory samples, has high sensitivity and specificity and is commercialized, but caution must be exercised in its interpretation.

Finally, molecular diagnosis can be used.

In medical practice, GC and India ink are used for clinical diagnosis, while Gram, gram, crystal violet, and nigrosin 9 stains can be used in laboratory and research practices .

OBJECTIVE

To study the capacity of different Cryptococcus genotypes for the formation of BP and to evaluate different staining techniques for their demonstration.

MATERIALS AND METHODS

It is a descriptive correlational study.

Strains: 6 strains of Cryptococcus spp sent by the mycology service of Hospital F. J Muñiz (CABA) previously genotyped were used .

For this, PCR-RFLP was used by amplifying the URA5 gene followed by enzymatic digestion by Sau 96l and Hha I.

RFLP (Restriction Fragment Length Polymorphic) patterns were assigned by comparison to standards obtained from reference strains (C. neoformans var. grubii : CBS 10085 VNI and CBS 10084 VNII; C. neoformans hybrid AD: CBS 10080 VNIII; C. neoformans var. neoformans : CBS 10079 VNIV; and C. gattii : CBS 10078 VGI; CBS 10082 VGII; CBS 10081 VGIII and CBS 10101 VGIV).

Biofilm: The 6 genotyped strains were subcultured in Sabouraud (SB) broth.

The cultures were shaken for their homogenization.

They were incubated for 48 hours; after which 100 uL of each one were taken and both were inoculated into two tubes of SB with DV as an abiotic surface inside.

The incubation was carried out for 24, 48, 72 hours and in 2 cases 1 week.

At the end of the incubation time provided for each one, the DVs were removed in a sterile manner, washed with sterile water and one of the sides was cleaned with filter paper. Subsequently, they were fixed by solling and stained: one with CV (0.5%) and the other with nigrosin (10% concentration) (Figure 1).

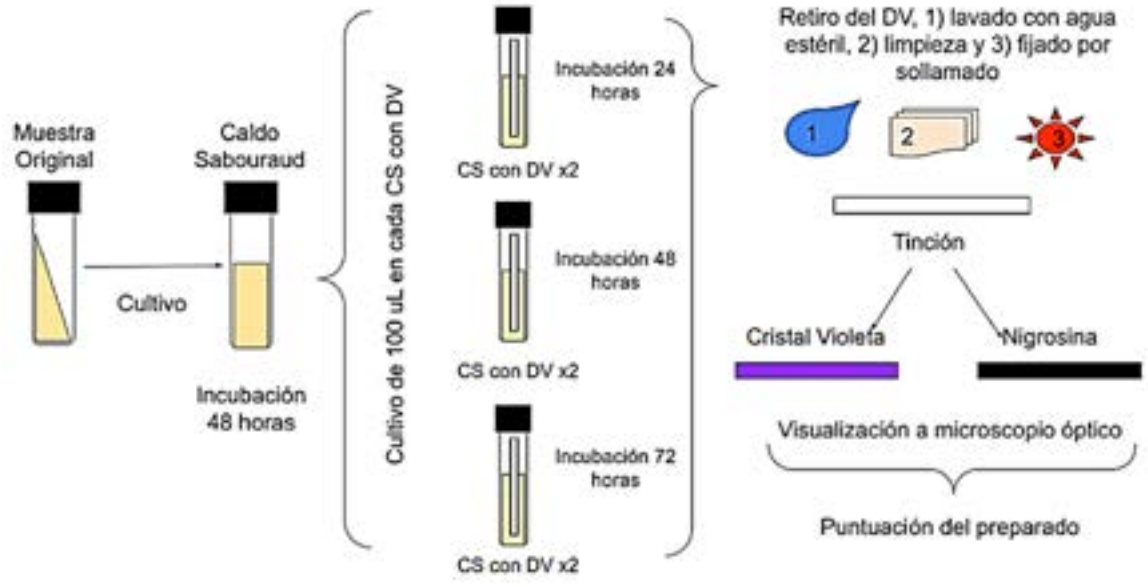
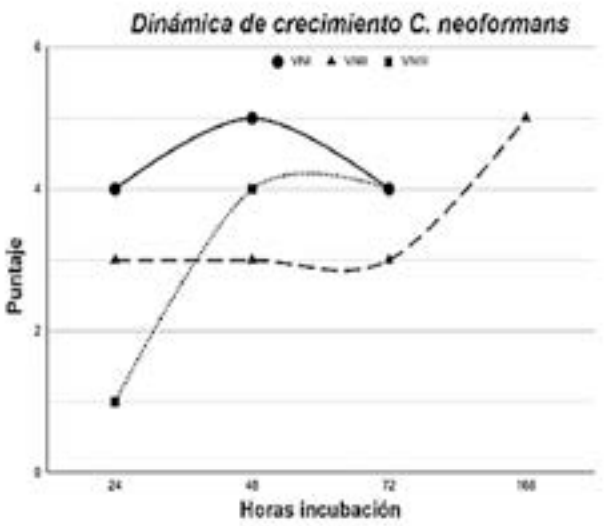


Figure 1: Processing of the samples. VGII and VNII were also cultured for 1 week



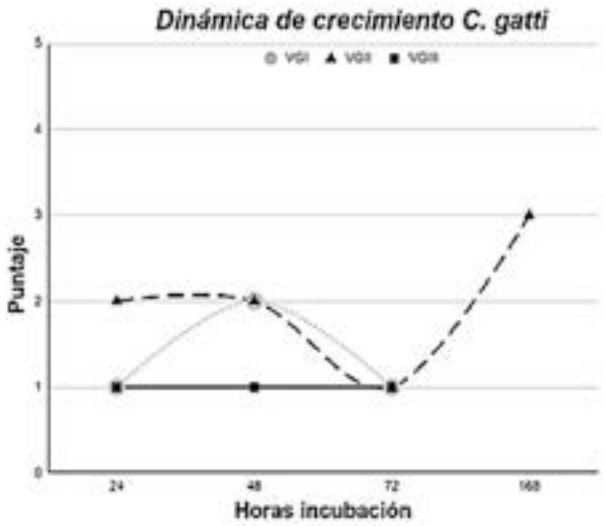


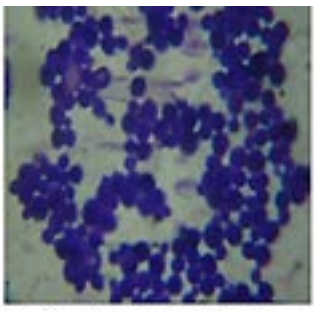
Fig. 2: Amount of accumulated cells per 1000x field.

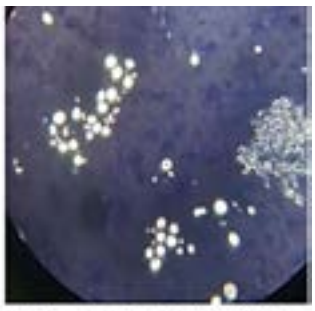
An optical microscope (OM) was used to observe them.
A score was assigned to the biofilms.
This takes into account the number of cells accumulated per 1000x field, assigning a score between 1 and 5 (Fig. 2).
Data collection: Camera and "Google apps for work".

RESULTS

BP growth dynamics:

The DVs of each strain were extracted at 24, 48, and 72 hours after their incubation at 36.5 °C for 48 hours of incubation.
The same was done after one week of culture with the VNII and VGII genotypes.
Microscopic observation: The score used in previous works was applied (Table 2).
At 24 hours it was observed that the VNI, VNII and VNIII genotypes gave a score of 4, 3 and 1; from 5, 3 and 4 at 48 hours and 4, 3 and 4 at 72 hours.
With the VGI, VGII and VGIII genotypes the scores were 1, 2 and 1 at 24 hours; 2, 2, 1 at 48 hours and 1, 1 and 1 at 72 hours.
After a week VNII and VGII obtained a score of 5 and 3 respectively (Figure 4).
In most genotypes, it was observed that BP formation decreased at 72 hours but increased at 7 days in VGII and VNII. The formation of hyphae in the VGI was also evidenced at 48 hours.
Staining selection: Figures 2 and 3 show the BP stained with CV and nigrosin respectively. There were no differences when comparing the scores of the stains.
Nigrosin allowed the visualization of the yeasts as "white spheres surrounded by a halo, corresponding to the capsule", staining with CV does not allow their visualization. Due to the ability to highlight the capsule, the former was chosen over the latter.





DISCUSSION

Cryptococcosis is a disease that has increased its prevalence in recent decades, concomitantly with the increase in immunosuppressive diseases, mainly the HIV pandemic since the 1980s, and it went from being an occasional disease, with 300 cases per year to a health emergency with approximately 1 million cases per year [8, 17] .
The diagnosis of the diseases is made through conventional studies [9, 17, 18].
Regarding stains, the most used to diagnose the presence of Cryptococcus spp in clinical samples are India ink and GC. However, for the study of BP we were able to verify that nigrosine turned out to be more effective compared to CV, a technique used routinely for the study of BP 19.
Nigrosin was chosen because it highlights the presence of the yeast capsule, the main virulence factor of the pathogen.
Facilitates visualization and differentiation with other yeast-like pathogens.
This is due to the hydrophobic nature of the solution, since the capsule, being composed of polysaccharides, has a hydrophilic nature that prevents the penetration of nigrosin [9,20].
Another of the advantages of the coloration would be the evaluation of the behavior of the capsule according to culture hours and Cryptococcus genotypes, an interes-

ting edge to investigate in the future.

BP are sessile communities of microorganisms adhered to a surface and embedded in an exopolymer that decreases the penetration of drugs, antibodies, and complement molecules [12, 15, 16] .

In the case of this yeast, it prevents the theoretical synergism between the antifungal and antibodies, giving rise to antagonism between the two [12, 21].

Antimicrobial treatment requires the proper approach of the drug to the microorganism that is denied by the BP.

In addition to this important resistance factor, there are structures that block its activity, which is repeated with antibodies. Let us remember that the capsule is an important factor of pathogenicity just as it occurs in the encapsulated bacteria.

Cryptococcus spp has a capsule rich in polysaccharides such as galactoxylomannan and mannoproteins, but glucuronoxylomannan (GXM) is the main component of the capsule and the target of antibodies and complement proteins [21,22].

Its porosity varies according to the distance from the cell wall, being greater towards the outside and less towards the inside.

This is what hinders the penetration of large molecules, such as those previously mentioned [23] .

The synthesis increases during infections and is thinner in the saprophytic forms [22].

Regarding antimicrobial resistance factors of this genus, the presence of melanin in its wall which decreases the action of amphotericin B and caspofungin [12, 16, 22].

All these factors would interfere with adequate therapy, which is of vital importance in serious infectious diseases.

In our country, amphotericin B is usually used as monotherapy for cryptococcosis, or in combination with fluconazole during treatment induction.

Unfortunately, the lack of importation and manufacturing of flucytosine makes another type of combination difficult [17, 24].

Amphotericin B has poor penetration of the blood-brain barrier, only passing through 4% of the plasma concentration [25].

The presence of BP would further block its penetration, like that of other drugs such as azoles and caspofungin.

According to several authors, the MICs for amphotericin B and caspofungin increase 4 to 8 times, while azoles such as fluconazole and voriconazole did not show any effect on them [24,25].

The increase in the MIC is accompanied by a greater risk of adverse effects such as hypokalemia, nephrotoxicity and anemia 17 that may force abandoning treatment and selecting other alternatives.

In cases of meningoencephalitis, reducing the CSF sterilization time is important since it reduces hospitalization time and the risk of acquiring nosocomial diseases.

worth emphasizing the importance of latent infections at the lung level by this microorganism, acquired mainly in childhood , 26 since it was documented that cryptococcosis from this focus presents greater resistance by forming titanic cells (which are polyploid giant cells, with great wall) isolated or in BP.

In them there is constant genetic exchange that adds to the exposure for years to antifungals the selection of yeasts resistant to them [6,7].

In this work we tried to determine the ability to form BP of Cryptococcus spp isolates previously genotyped.

As expressed once the diagnosis is made, it is necessary to establish the appropriate antifungal treatment that may fail, but the reason for this failure is not known with certainty.

This could be due to the formation of BP.

C. neoformans strains obtained higher scores than those of C. gatti, always higher than 3, so they would be able to form more robust BP, with the consequent possibility of therapeutic failure.

C. gatti must possess other virulence factors that favor infection, having a deficit in adhesion and/or BP formation when compared to C. neoformans .

This would also explain the reason for the notification of more cases of meningoencephalitis caused by C. neoformans than by C. gatti .

Knowing precisely the genotype of Cryptococcus recovered from an infection and its ability to form BP could predict the course and prognosis of therapy in HIV-positive patients.

This was one of the initial objectives that could not be achieved due to the appearance of the pandemic.

In addition, more studies are needed with antifungals that have greater BP penetration, as demonstrated with anidulafungin against C. albicans [27].

Conclusion, C. neoformans is an opportunistic pathogen with important virulence factors, among which we must certainly mention its ability to form BP. As demonstrated, genotyping or at least the use of techniques that allow a more precise identification of the isolate, including the formation of BP from clinical samples, would be necessary for the success of antifungal therapy.

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