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Cryptococcus spp., Identification and Susceptibility to Antifungals in Isolated in Laboratory of Reference in The State of Rio Grande Do Sul, Brazil

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Abstract

Introduction: The *Cryptococcus* spp., fungus with opportunistic behavior, present in environmental sources, bird excreta, remains of trees, among others. It is inhaled by the individual, causing infection that can spread to other organs and systems with tropism through the central nervous system, causing meningoencephalitis. It is estimated that *C. neoformans* causes more than one million new cases of cryptococcosis per year, about 625,000 deaths. Meningoencephalitis, the most common clinical manifestation, is estimated to cause 120,000 to 240,000 deaths per year worldwide. Methods: This study was conducted from January 2012 to June 2019. Biological materials were processed, mainly the cerebrospinal fluid of patients with suspected cryptococcosis. All the phenotypically identified isolates were confirmed by the Polymerase Chain Reaction (PCR) molecular technique. The antifungals fluconazole, itraconazole and amphotericin B were also tested to verify the sensitivity profile of the isolates. Conclusions and discussion: In this study it was possible to observe and confirm a higher prevalence of *Cryptococcus neoformans* species than *C. gattii*. Azole resistance was also observed by the fungus *Cryptococcus* spp., suggesting a possible synergism of azoles with anti-inflammatory substances, but further studies are needed both *in vitro* and *in vivo*. The imminent biological risk from the presence of the two fungal species in the environment, including the confirmation of antifungal resistant strains, corroborates the need for continuous environmental monitoring, including the presence of new pathogenic species and consequently how to conduct treatment in the various clinic presentations by this yeast.

Keywords

Cryptococcus spp., identification, susceptibility, Rio Grande do Sul.

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INTRODUCTION:

Cryptococcosis is a fungal infection caused by the yeast of the genus *Cryptococcus*, whose most frequent species are *C. neoformans* and *C. gatti* (7). They are responsible for systemic infections that especially affect immunosuppressed individuals, such as newborns, transplanted individuals and those with acquired immunodeficiency syndrome (AIDS) (8). As an etiological agent, it is estimated that *C. neoformans* causes more than one million new cases of cryptococcosis per year, with about 625,000 deaths (6). *Cryptococcus* spp. is a fungus with opportunistic behavior, has the airway as a gateway causing pulmonary infection and later spreading to other organs and systems, among them the tropism by the central nervous system, causing meningoencephalitis (1, 6, 20).

As for cryptococcal meningitis, the most common clinical manifestation, it is estimated to result in 120,000 to 240,000 deaths per year worldwide (22). This microorganism is found in environmental sources, especially where there is contamination by pigeon excreta and also in substrates such as tree hollows, hornet nests, bat feces, among others (13,21) and with higher prevalence in tropical and subtropical regions (25).

Worldwide and especially in Latin America, *C. neoformans* causes more than 90% of cases of cryptococcosis (3), predominantly in patients with human immunodeficiency virus (HIV), whereas *C. gattii* is more likely to infect immunocompetent individuals (10,13). Latin America has been identified as the region of the American continent with the highest number of cases per year, with approximately 5,300 cases. Of these, Brazil and Colombia were the countries with the highest incidence, between 1,001 and 2,500 cases of cryptococcosis (4).

In Rio Grande do Sul (RS) reports indicate a higher prevalence in the metropolitan region of Porto Alegre (RS), with males and immunosuppressed individuals being the most affected by the disease, with the main immunosuppression factor being AIDS (6,16).

The golden standard treatment for cryptococcosis is the combination of amphotericin B and 5-flucytosine (11). However, due to its high cost, 5-flucytosine is not present in therapeutic protocols in several countries, giving rise to azole drugs such as fluconazole and itraconazole (26). However, azoles have been showing ineffectiveness due to the development of resistant strains (9). Moreover, although resistance to amphotericin B polyene is considered rare and has a good activity spectrum, this drug has restricted use due to nephrotoxicity problems (11,30).

Currently, few antifungals are commercially available and the development of new drugs has not accompanied the development of resistant strains (12). Combination therapy with two or more antifungals has been tested in an attempt to reduce antifungal resistance and decrease the toxicity of each drug, but its side effects should still be carefully evaluated (10, 29). Therefore, studies of *in vitro* association with non-antifungal agents and antifungal drugs have been suggested but it is still necessary to delineate *in vitro* assays and consequently clinical trials (10, 29).

The present study aimed to verify the presence of *Cryptococcus* spp. species isolated in a reference laboratory of the State of Rio Grande do Sul (LACEN-RS), as well as to evaluate the susceptibility profile to antifungals. The research project was approved by the research ethics committee under number 29595.

METHODOLOGY:

The data obtained in this study come from the Mycology section of the Central Laboratory of the State of Rio Grande do Sul (LACEN-RS) from January 2012 to June 2019. In LACEN-RS, biological materials were processed, mainly the cerebrospinal fluid of patients with suspected cryptococcosis. The mycology department of LACEN-RS, when receiving the patient's biological sample, sowed in a culture medium Sabouraud dextrose agar with chloramphenicol without cycloheximide, being incubated at a temperature of 25 to 30ºC, and observed daily for seven days. After the smooth, moist colonies indicative of *Cryptococcus* spp. were submitted to phenotypic tests of urease which presents pink coloration for *Cryptococcus* spp. (urease positive) and no change in medium color for *Candida* spp. (urease negative). In positive cases for *Cryptococcus* spp. the canavanine and glycine test were used, with a canavanine glycine bromothymol blue (CGB) medium used as confirmation to *Cryptococcus gattii* and *Cryptococcus neoformans* species.

C. gattii is glycine resistant and hydrolyses L-canavanine, therefore degradation occurs in ammonia, altering the pH of the medium and changing the color of the culture medium to cobalt blue. *C. neoformans* is inhibited by glycine and does not hydrolyze L-canavanine, does not alter the pH of the medium and therefore the culture medium does not change color. All the phenotypically identified by LACEN-RS isolates were then sent to the UFRGS Faculty of Pharmacy Mycology Laboratory (Biomicolab) to be confirmed by the Polymerase

Chain Reaction (PCR) molecular technique through the primers
CNa-70S (5'-ATTGCGTCCACCAAGGAGCTC-3') and CNa-70A (5'-ATTGCGTCCATGTTACGTGGC-3').
For susceptibility tests, the antifungals fluconazole (FLC), itraconazole (ITC) and amphotericin B (AMB) were tested. According to the recommendations of the Clinical and Laboratory Standards Institute (CLSI) (2), the FLC stock solution (Metrochem Api Private Limited, India) was prepared in distilled water. ITC (Metrochem Api Private Limited) and AMB (Metrochem Api Private Limited) stock solutions were prepared in DMSO (Nuclear, Brazil). For the experiments, the compounds were diluted in RPMI 1640 medium (Sigma-Aldrich) to obtain a maximum concentration of 2% Dimethyl sulfoxide (DMSO).
Minimum inhibitory concentrations (MICs) of antifungal agents were determined in duplicate by the micro dilution in broth method according to protocol M27-A3 (CLSI, 2008). Serial dilutions were made twice in RPMI 1640 medium (Sigma-Aldrich) buffered with MOPS (Sigma-Aldrich) and the concentration ranges tested were: 0.125 - 64 µg/ml FLC, 16 - 0.0312 µg/ml of ITC and 0.0312 - 16 µg/ml AMB. MICs values were defined as the lowest concentration of compounds in which the tested microorganisms showed no visible growth (AMB) or reduced 50% or 90% growth (FLC and ITC) within 72 h.

RESULTS AND DISCUSSION:
The period studied corresponds from January 2012 to June 2019 totalizing 139 isolates of Cryptococcus spp. The total number and confirmed cases per year are described in Figure 1.

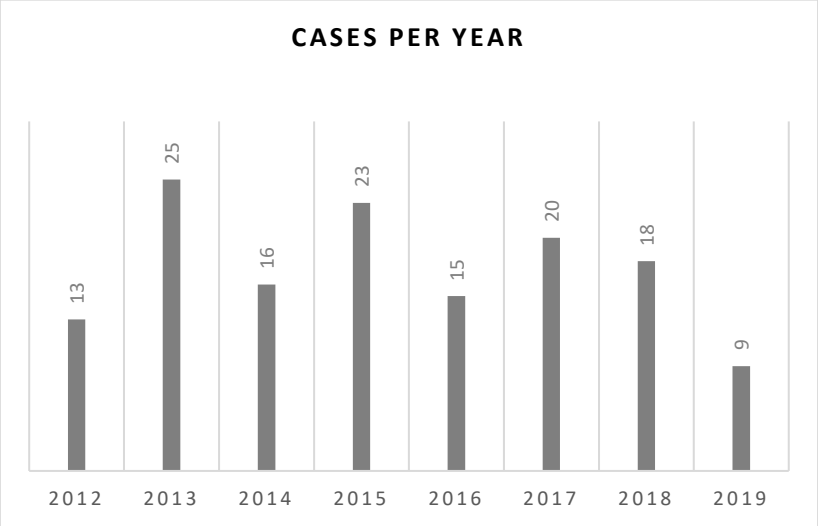

Figure 1: Number of *Cryptococcus* spp. isolates per year, isolated at LACEN- RS in the period of January 2012 to June 2019.

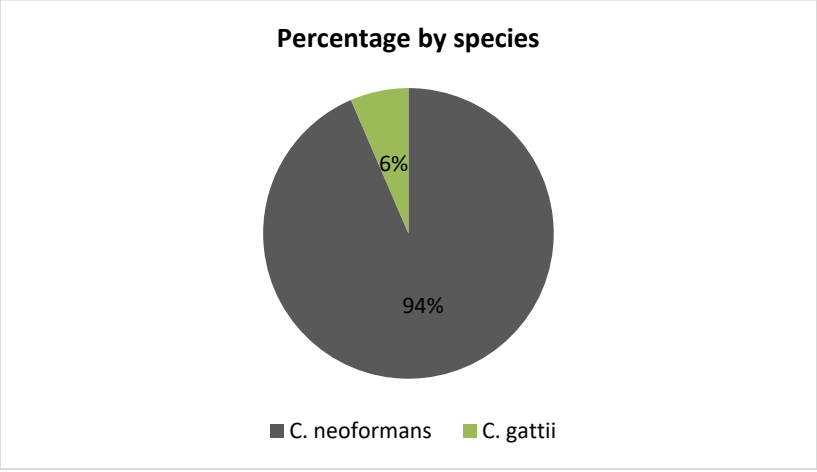

Figure 2: Percentage between the two species of confirmed *Cryptococcus* spp., *C. neoformans* and *C. gattii*.

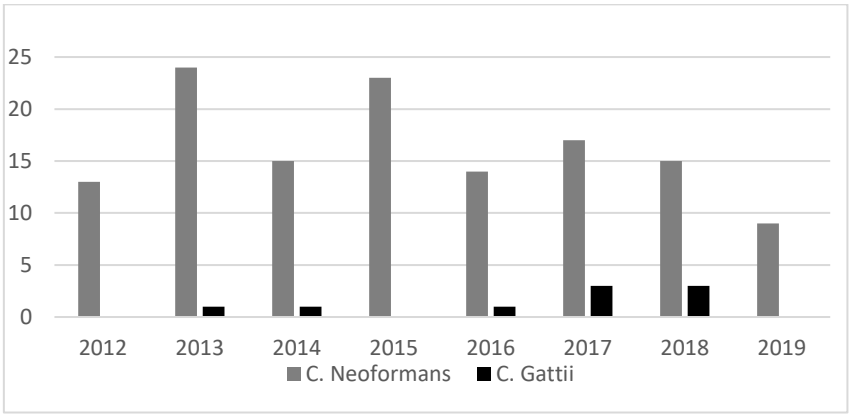

Figure 3: Total *Cryptococcus* species isolated and identified during the period, annually.

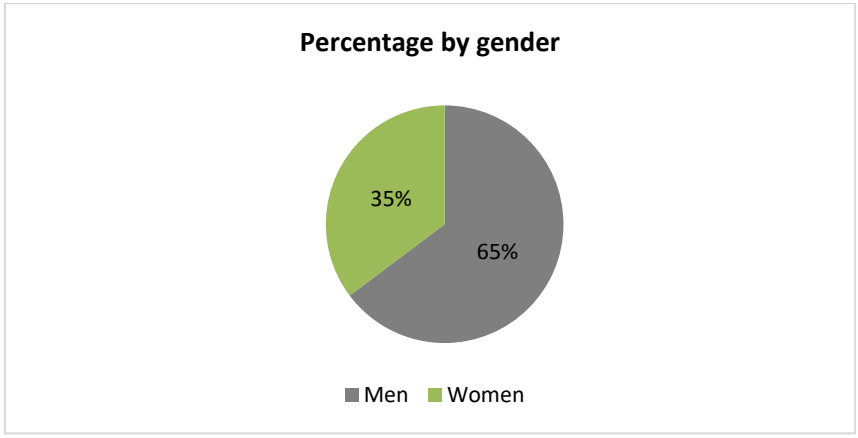

Figure 4: Percentage difference in the number of cases of *Cryptococcus* spp. by gender

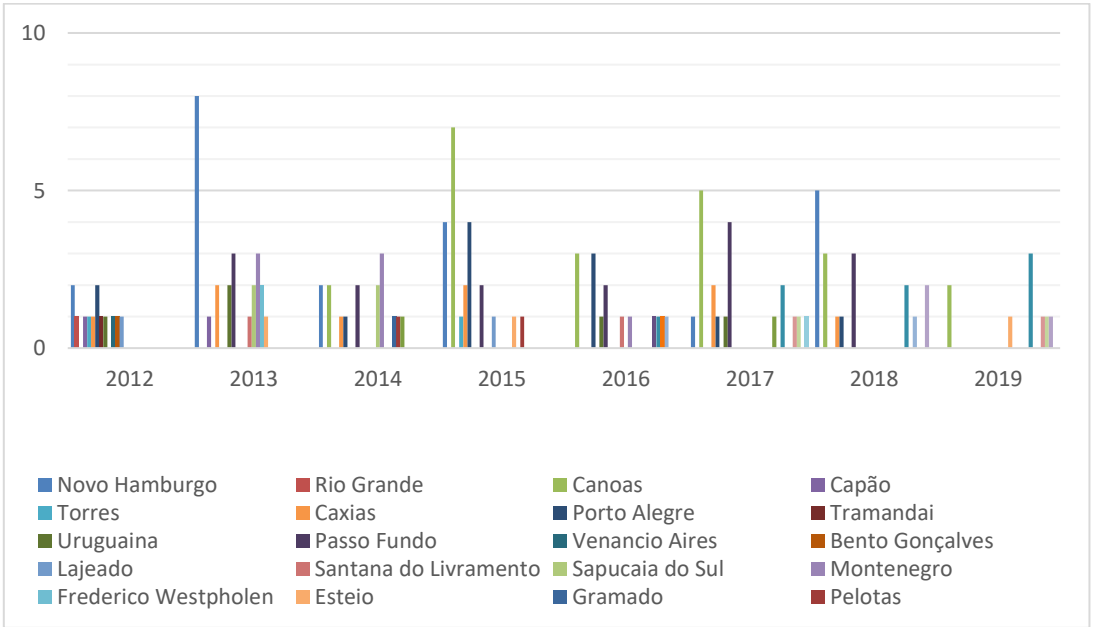

Figure 5: Annual distribution of *Cryptococcus* spp. isolates in the cities of the state of Rio Grande do Sul during the period of the study.

Of the 139 confirmed isolates of *Cryptococcus* spp., 25 isolates of *Cryptococcus neoformans* were randomly selected to check the susceptibility profile to the fluconazole, itraconazole and amphotericin B antifungals. Results were expressed as susceptibility profile (µg/ml) expressed in ranges of variation of minimum and maximum values of Minimum Inhibitory Concentration (MIC ranges), geometric mean (GM), MIC_{50} (MIC value that inhibits 50% of the isolates) and MIC_{90} (MIC value that inhibits 90% of the isolates) for the antifungals fluconazole (FLC), itraconazole (ITC) and amphotericin B (AMB), the results are presented in Table I.

Table I. Susceptibility profile (µg/ml) of twenty-five isolates of *Cryptococcus neoformans* to antifungal agents expressed in ranges of variation of minimum and maximum MIC values (MIC ranges), geometric mean (GM), MIC_{50} (MIC value that inhibits 50% of the isolates) and MIC_{90} (MIC value that inhibits 90% of the isolates).

Agents	MIC range (µg/ml)	GM (µg/ml)	MIC_{50} (µg/ml)	MIC_{90} (µg/ml)
ITC	0.0312 - 16	0.66	1	1
FLC	0.25 – 8	1.74	2	4
AMB	0.5 – 16	6.17	4	16

The number of confirmed isolates of *Cryptococcus* spp. at the Central Laboratory of the State of Rio Grande do Sul (LACEN-RS) from January 2012 to June 2019 were 139 cases from 29 cities of the State of Rio Grande do Sul, with an average of 17.4 (12.5%) cases per year. In 2013, it was the year with the highest number of isolates, 25.0 (17.9%) followed by 2015 with 23.0 (16.5%) cases of isolation and identification of *Cryptococcus* spp. **(Figure 1)**. These data in relation to the annual isolates agrees with the literature when observed by regions or countries (4).

We can observe that it had been a prevalence of *Cryptococcus neoformans* comparing to *Cryptococcus gattii* in the total of isolates during the period of study **(Figure 2).** In the present study 94% of the cases have *Cryptococcus neoformans* and 6% have *Cryptococcus gattii* as the etiological agent, corroborating with the literature, with *Cryptococcus neoformans* causing over 90% of the cryptococcosis cases worldwide (1). In this study, 2013 was the year with the largest number, 24 (17.3%) of *Cryptococcus neoformans* isolates, with 2017 and 2018 as the ones with the highest number of isolates, 3 (2.2%) of *Cryptococcus gattii* **(Figure 3)**. This result corroborates with epidemiological studies from other regions of the world (4).

Based on literature findings, including data published in the Brazilian Cryptococcosis Consensus in 2008 (3,4), 78% of confirmed cases of infection are male, as reported by the Ibero American Cryptococcal Study Group in 2003 (17), which refers to the incidence of 5.1 times higher in men than in women. In the present study, 65% of confirmed cases were in male patients **(Figure 4)**. It is possible that female hormones play an important role in the body's defense against *Cryptococcus* spp., as previously established in paracoccidioidomycosis. In studies with rats, females infected with *Cryptococcus neoformans* had higher levels of TNF-α and IFN-γ in the spleen and blood compared to males (6,16,18).

Of the 139 isolates of *Cryptococcus* spp., 56 (40.3%) were predominant in the cities of the metropolitan region of Porto Alegre, where 22 (39.3%) in the city of Novo Hamburgo, 22 (39.3%) in Canoas and 12 (21.4%) in Porto Alegre **(Figure 5)**. Regarding the annual distribution by city of the state of Rio Grande do Sul, the years with the largest number of isolates were 25 (18.0%) in 2013 and 23 (16.5%) in 2015, confirming again that Novo Hamburgo was the city with the largest number of isolates, 8 (5.6%) in 2013, and Canoas had the highest number, 7 (5.0%) in 2015, followed by Porto Alegre with 4 (2.9%), results that can be justified due to being cities of the metropolitan region of Porto Alegre, with a high concentration of pigeons, large urban centers and consequently greater contact of individuals with the infecting elements of the fungus. However, this factor should be better evaluated including factors such as the patient's occupation and origin that may contribute to the acquisition of the disease. These data corroborate with previous studies realized in the state of Rio Grande do Sul (18,19,25).

Table I shows that isolates with low sensitivity to antifungal agents were found. These fungi in the environment, including areas of high concentration of people, may pose as a biohazard, and the presence of antifungal resistant strains may lead to a different way of treating the pathologies caused by this yeast (20). Fuentefria et al. (2017) (5) describes that the limited efficacy and difficulty of introducing new antifungals in the market should be considered corroborating the recommendation of using drug

combination as a therapeutic strategy for the treatment of potentially fatal invasive fungal infections. Other studies also reinforce this perspective and by detecting synergism between anti-inflammatory drugs with azoles for both *Candida albicans* and *Cryptococcus neoformans*, increasing the susceptibility of isolates to these antifungal agents (21,26), but in the present study such recommendations described by other authors have not been tested, which leads to a future focus on isolation and identification of species of *Cryptococcus* spp., as well as to monitor the susceptibility profile of these potentially severe and often fatal mycosis agents.

CONCLUSIONS:

From January 2012 to June 2019, it was possible to observe and confirm a higher prevalence of isolates of the species *Cryptococcus neoformans*, and its higher incidence in men, which corroborates the data already described in the literature.

As for the region with the largest number of isolates, the metropolitan region of Porto Alegre stands out, in the cities of Novo Hamburgo, Canoas and Porto Alegre, cities with high population density and the presence of a large number of pigeons.

The results of this study confirm that azole resistance already exists for the fungus *Cryptococcus* spp. when used in cryptococcosis therapy. However, the synergism of azoles with anti-inflammatory substances need further studies both *in vitro* and *in vivo* in clinical situations to approve the beneficial effects of this combination.

The imminent biological risk from the presence of these two fungal species present in the environment, including the confirmation of antifungal resistant strains corroborates the need for continuous environmental monitoring, including the presence of new pathogenic species and consequently the way to conduct treatment in the various clinical presentations caused by this year.

REFERENCES:

1. Chen, S., Yan, H., Zhang, L., Kong, W., Sun, Y., Zhang, W., Chen, Y., Deng, A. (2015). *Cryptococcus neoformans*. Infectionand Immune Cell Regulation in Human Monocytes. *Cell Physiol Biochem*, 37:537-547.
2. Clinical And Laboratory Standards Institute (CLSI). Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard – Third Edition. CLSI Document M27-A3. *Clinical Laboratory Standards Institute*, Wayne, PA, USA, 2008.
3. Consenso em criptococose. (2008). *Rev Soc Bras Med Trop*, Uberaba Sept./Oct, 41(5):524-544.
4. Firacative, C., Lizarazo, J., Illnait-Saragoça, M.T., Castañeda, E. (2018). O status da criptococose na América Latina. *Mem Inst Oswaldo Cruz*, Rio de Janeiro, 113(7):1-15.
5. Fuentefria, A.M., Pippi, B., Dalla Lana, D.F., Doanto, K.K., De Andrade, S.F. (2017). Antifungals discovery: an insight into new strategies to combat antifungal resistance. *Letters in Applied Microbiology*, 66(1):2-13.
6. Geraldo, L.F. A Criptococose em pacientes com hiv/aids, Trabalho de Conclusão apresentado ao Curso de Pós-graduação em Microbiologia da Universidade Federal de Minas Gerais como requisito à obtenção do título Especialista.; Belo Horizonte, 2017.
7. Gomes, F.S., Sarmento, D.N., Santo, E.P.T.E., Silva, S.H.M. (2010). Quimiotipagem e caracterização fenotípica de Cryptococcus isolados em Belém, Estado do Pará, Brasil. *Rev Pan-Amaz Saude*, 1(4):43-49.
8. Grimaldi, M., De Rosa, M., Di Marino, S., Scrima, M., Posteraro, B., Sanguinetti, M., Fadda, G., Soriente, A., D'Ursi, A.M. (2010). Synthesis of new antifungal peptides selective against *Cryptococcus neoformans*. *Bioorganic & Medicinal Chemistry*, 18:7985–7990.
9. Gullo, F.P., Rossi, S.A., Sardi, J., Teodoro, V.L.I., Mendes-Giannini, M.J.S., Fusco-Almeida, A.M. (2013). Cryptococcosis: epidemiology, fungal resistance, and new alternatives for treatment. *Eur J Clin Microbiol Infect Dis*, 32:1377–1391.
10. Hatipoglu, N., Hatipoglu, H. (2013). Combination antifungal therapy for invasive fungal infections in children and adults. Expert Rev. *Anti Infect. Ther*, 11:523-535.
11. Kagan, S., Ickowicz, D., Shmuel, M., Altschuler, Y., Sionov, E., Pitusi, M., Weiss, A., Farber, S., Domb, A., Polachecka, I. (2012). Toxicity mechanisms of amphotericin B and its neutralization by conjugation with arabinogalactan. *Antimicrobial Agents and Chemotherapy*, 56:5303-5611.
12. Lima, F.R., Lopes, A.F.B., Lyra, L., Peron, I.H., Taguchi, H., Mikami, Y., Kamei, K., Moretti, M.L., Schreiber, A.Z. (2016). Evaluation of antifungal combination against *Cryptococcus* spp. *Mycoses*, 59:585–593.
13. Lima, A.C.A.G.S., Shizuo, I., Maksymczuk, D.R.D. (2017). Neurocriptococose por *Cryptococcus gattii* resistente a fluconazol em imunocompetente. *Rev Soc Bras Clin Med*,15(2):124-6.
14. Liu. S., Houb. Y., Chenc. X., Gaoa, Y., Li, H., Sund, S. (2014). Combination of fluconazole with non-antifungal agents: A promising approach to cope with resistant Candida albicans infections and insight into new antifungal agent discovery. *Internat J Antimicrob Agents*, 43:395-402.
15. Lopes, J.O., Costa, J.M., Streher, L.A., Clock, C., Pinto, M.S., Alves, S.H. (1997). Criptococose não associada à AIDS no Rio Grande do Sul: relato de oito casos e revisão da literatura sul-riograndense. *Rev Soc Bras Med Tropical*, 30(5):369-372.
16. Lortholary, O., Improvisi, L., Fitting, C., Cavaillon, J.M., Dromer, F. (2002). Influence of gender and age on course of infection and cytokine responses in mice with disseminated *Cryptococcus neoformans* infection. *Clinical Microb and Infection*, 8(1):31-7.

17. Meyer, W., Castañeda, A., Jackson, S., Huynh, M., Castañeda, E. (2003). Tipagem molecular de isolados Ibero-Americanos de *Cryptococcus neoformans*. *Emerg Infect Dis*, 9(2):189-95.
18. Mezzari, A., Wliebbelling, A.M.P., Freitas, G.S.O., May, G.G., Albé, G.C., Filik. H.P., Portich, J.P., Kissmann, N., Behar, P., Vilela, R.M.M. (2013). Criptococose em um Hospital Público de Porto Alegre: dados epidemiológicos. *J Infect Control*, 2(3):135-139.
19. Mezzari, A., Wliebbelling, A.M.P., Wenczenovicz, C., Souza, C.D.A., Freitas, G.S.O., Barboza, L.D., Pena, L.D., Kissmann, N., Portich, J.P., Carneiro, L.C., Behar, P.R.P. (2014). Presença do *Cryptococcus* spp. nas excretas de pombos nos arredores de Hospitais de Porto Alegre. *Rev Panam Infectol*, 16(3):153-160.
20. Mezzari, A., Wliebbelling, A.M.P., May, G.G., Albé, G.C., Filik, H.P., Esquerdo, D., Fidalgo, N.C.G., Behar, P.R.P. (2015). Presença e susceptibilidade aos antifúngicos do *Cryptococcus* spp. em excretas de pombos nos arredores dos grandes hospitais de Porto Alegre. *Rev AMRIGS*, Porto Alegre, 59 (3):204-208.
21. Ogundeji, A.O., Pohl, C.O., Sebolai, O.M. (2016). Repurposing of Aspirin and Ibuprofen as Candidate Anti-Cryptococcus Drugs. *Antimicrobial Agents and Chemotherapy*, 60: 4799-4808.
22. Prates, R.A., Fuchs, B.B., Mizuno, K., Naqvi, Q., Kato, I.K., Ribeiro, M.R., Mylonakis, E., Tegos, G.P., Hamblin, M.R. (2013). Effect of Virulence Factors on the Photodynamic Inactivation of *Cryptococcus neoformans*. *PLoS ONE*, 8(1): e54387.
23. Queiroz, J.P.A.F., Sousa, F.D.N., Lage, R.A., Izael, M.A., Santos, A.G. (2008). Criptococose – uma revisão bibliográfica. *Acta Veterinaria Brasilica*, 2(2):32-38.
24. Rajasingham, R., Smith, R.M., Park, B.J., Jarvis, J.N., Govender, N.P., Chiller, T.M., Denning, D.W., Loyse, A., Boulware, D.R. (2017). Global burden of disease of HIV-associated cryptococcal meningitis: an updated analysis. *Lancet Infect Dis*, 17:873-881.
25. Reolon, A., Peres, L.R.R., Mezzari, A. (2004). Prevalência de Cryptococcus neoformans nos pombos urbanos da cidade de Porto Alegre, Rio Grande do Sul. *J Bras Patol Med Lab*, 40(5):293-8.
26. Ricardo, E., Oliveira, S.C., Dias, A.S., Guerra, J., Rodrigues, A.G., Pina-Vaz, C. (2009). Ibuprofen reverts antifungal resistance on *Candida albicans* showing overexpression of CDR genes. *FEMS Yeast Res*, 9:618–625.
27. Silva, M.R., Lima, C.M.O., Schechtman, R.C., Trope, B.M., Carneiro, S. (2012). Systemic mycoses in immunodepressed patients (AIDS). *Clinics in Dermatology*, 30:616-627.
28. Smith, K.D., Achan, B., HupplerHullsiek, K., McDonald, T.R., Okagaki, L.H., Alhadab, A.A., Akampurira, A., Rhein, J.R., Meya, D.B., Boulware, D.R., Nielsen, K. (2015). Increased antifungal drug resistance in clinical isolates of *Cryptococcus neoformans* in Uganda. *Antimicrobial Agents and Chemotherapy*, 59:7197–7204.
29. Venturini, T.P., Rossato, L., Spader, T.B., Tronco-Alves, G.R., Azevedo, M.I., Weiler, C.B., Santurio, J.M., Alves, S. H. (2011). In vitro synergisms obtained by amphotericin B and voriconazole associated with non-antifungal agents against *Fusarium* spp. *Diagnostic Microbiol and Infect Disease*, 71:126–130.
30. Xie, J.L., Polvi1, E., Shekhar-Guturja, T., Cowen, L. (2014). Elucidating drug resistance in human fungal pathogens. *Future Microbiol*, 9:523-542.