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Diversity of potencial pathogenic *Cryptococcus* species isolated from environment in country side São Paulo state, Brazil

Diversidade de espécies de Cryptococcus potencialmente patogênicas isoladas do meio ambiente no interior do estado de São Paulo, Brasil

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ABSTRACT

Cryptococcosis is a systemic disease caused by two species. C. neof*ormans* species complexes are associated with cases of cryptococcosis in immunosuppressed patients, being commonly isolated from the soil and birds feces. *C. gattii* species complexes affects mainly immunocompetent individuals and is often isolated in native or exotic tree species in state of biodegradation. This study aimed to perform the environmental mapping of pathogenic species of the genus *Cryptococcus* in tree samples of public places of the city of Birigui, São Paulo. The samples were collected from regions with greater traffic in this city. After processing, morphophysiological, biochemical, carbon and nitrogen assimilation, URA5 PCR and RFLP with double enzymatic digestion tests were performed to characterize the *Cryptococcus* species/species complexes. The percentage of tree colonization by the *C. gattii* VGII was 5%, for *C. neoformans* VNIV was 1%, and was found 6% of *C. laurentii* and 3% of *C. albidus* species. These results suggest that there are a micro-foci and diversity of pathogenic *Cryptococcus* species in arboreal environment locations investigated in this study.

Keywords: [*Cryptococcus gattii*; *Cryptococcus neoformans*; *Cryptococcus laurentii*.; Brazil ;Trees]

RESUMO

Criptococose é uma doença sistêmica causada por duas espécies. O complexo de espécies de *C neoformans e*stão associados a casos de criptococose em pacientes imunossuprimidos, sendo comumente isolados do solo e fezes de aves. Os complexos de espécies de C. gattii afetam principalmente indivíduos imunocompetentes e são freqüentemente isolados em espécies arbóreas nativas ou exóticas em estado de biodegradação. Este estudo teve como objetivo realizar o mapeamento ambiental de espécies patogênicas do gênero Cryptococcus em amostras de árvores de logradouros públicos da cidade de Birigui, São Paulo. As amostras foram coletadas nas regiões de maior tráfego desta cidade. Após o processamento, testes morfofisiológicos, bioquímicos, de assimilação de carbono e nitrogênio, URA5 PCR e RFLP com dupla digestão enzimática foram realizados para caracterizar os complexos espécies de *Cryptococcus*. A porcentagem de colonização das árvores por *C. gattii* VGII foi de 5%, para *C. neoformans* VNIV de 1%, 6 % *de C. laurentii* e 3% de *C. albidus.* O estudo sugere que há microfocos e diversidade de espécies patogênicas de Cryptococcus nas localidades do ambiente arbóreo investigadas neste estudo.

Palavras-chave:[*Cryptococcus gattii*; *Cryptococcus neoformans*; *Cryptococcus laurentii*.;Brasil ;Árvores]

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INTRODUÇÃO

Cryptococcosis is a systemic mycosis, which affects humans and animals, mainly mammals, rarely manifesting itself in birds. In humans, its most frequent manifestation is meningoencephalitis, while in animals it can present different conditions, such as infection of the nasal cavity, lungs, eyes, skin, and central nervous system (REFAI *el al*, 2017, YAN *et al*.2002).

The infection occurs by inhaling basidiospores of pathogenic species of the genus Cryptococcus (BRITO-SANTOS *et al*, 2015). These cells can remain dormant for a long time, and subsequently activated, affecting both immunocompetent and immunosuppressed individuals (SUN *et al*, 2009, MARUYAMA *et al*, 2019). It is estimated that 223,100 human cases occur annually in the world, with a mortality rate of 81% (Bentes *et al*, 2019). The main infectious agents belong to the species complexes *C. neoformans* and *C. gattii*, and there are other species of the genus capable of causing cryptococcosis, but still lacking in reported cases to date (HAGEN *et al*, 2016).

Despite the current taxonomic review, based on phylogenetic analysis of strains isolated from various locations around the world, proposing the separation of the species *C. gattii* and *neoformans* into five and two different species, respectively (Hagen *et. al* 2015), its is recognized that these new species belong to two species complexes, each composed of four major molecular types, with VNI, VNII, VNIII and VNIV belonging to the *C. neoformans* species complexes and VGI, VGII, VGIII and VGIV to the *C. gattii* species complexes (KWON-CHUNG *et al.*, 2017). The *C. neoformans* and *C. gattii* species complexes act on their hosts in different ways (MARUYAMA *et al*, 2019). Infections caused by the *C. neoformans* species complexes are opportunistic, commonly in urban environments and generally associated with conditions of immunosuppression (ANDRADE-SILVA *et al.*, 2018), while *C. gattii* species complexes are related to immunocompetent hosts, both of them can be fatal to the host (Brito-Santos *et al*, 2015). Although these fungi no need for a host for survival (GERSTEIN e NIELSEN, 2017), it is necessary to consider that not only animals isolates are important, but also the environment they inhabit. Cryptococcosis affects a wide range of living beings (REFAI *et al.*, 2017; SINGH *et al*, 2018), both wild and companion animals are important sentinels for human risk indicators, as they are always in contact with the environment (ACHESON *et al*.,2018). Possibly animals and humans are infected by the same natural

foci, although there is no report of mammal-mammal transmission to date (SINGH *et al*, 2018), the permanence and viability of the fungus in the environment stand out, such as its dissemination by the action of the wind, carrying the propagules to other adjacent excreta and present in the soil, thus considering factors relevant to the formation of microfocuses and the expansion of risks of environmental dissemination (CASADEVALL e PERFECT *et al*., 1999).

In their natural habitat, species of the genus *Cryptococcus* can be found in various organic substrates in a saprophytic form, often being related to rich sources of nitrogen (WATKINS *et al.*,2017). The species of the *C. neoformans* complexes are more associated with dry excreta of birds and soil, also being reported in guano from pigeons in urbanized regions, bats, and birds in captivity (VIEILLE *et al*.,2018; COLOMBO *et al.*,2015; TENCATE *et al.*, 2012; FILIÚ *et al*.,2002). Differently, the *C. gattii* complexes are associated with decaying plant materials, bark, flowers, and leaves of trees being isolated in several tree species in different countries (BENTES *et al*., 2019;WATKINS *et al.*,2017; HERKERT *et al*.,2017).

Studies carried out after the outbreaks that occurred in 2000 in Canada suggest that the global environmental distributions of these species complexes are changing, but the lack of environment isolates makes it impossible for a reliable prediction (ACHESON *et al.*,2018).

The characterization of the species distributed in the environment is important for prophylaxis and prevention of outbreaks, since the infection occurs through the inhalation of the pathogen disposed of in the environment and each complex of species acts differently in its host (BENTES *et al*., 2019; GERSTEIN and NIELSEN,2017). The differentiation of the molecular types is necessary to verify the complex patterns of species, thus enabling a greater understanding of their geographic distribution, and clinical involvement in humans and animals, and subsequently, enabling a better understanding of possible virulence and drug resistance genes (VIEILLE *et al*.,2018; MARUYAMA *et al*, 2019). Therefore, This work aimed to carry out the environmental mapping of pathogenic species of the genus *Cryptococcus* in environmental samples from trees in public places in Birigui, São Paulo.

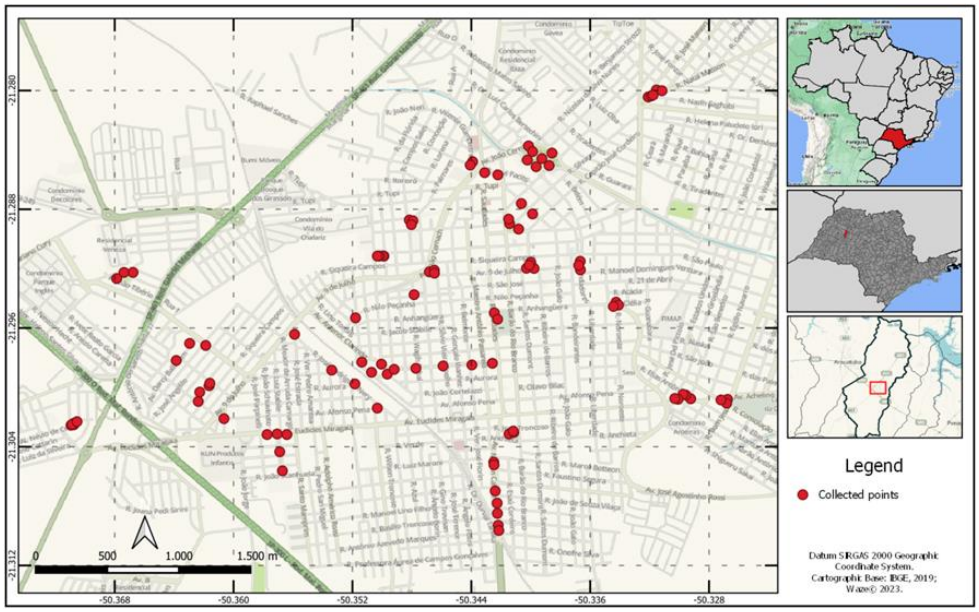
MATERIAL AND METHODS

Birigui is a municipality located in the northwest region of the state of São Paulo. It is geographically located at a latitude of 21º 16' 53" south and longitude of 50º 19' 35" west, being 11 kilometers from the city of Araçatuba and 521 kilometers from the capital, lying 400 meters above sea level, with estimated population of 126,094 inhabitants, according to IBGE/2021. Its climate is tropical, with occasional droughts in winter, with an average rainfall of 1229mm and an average annual temperature of 23.6ºC (CEPAGRI,2016).

To define the collected points, the geographic map of the municipality was used, considering its urban perimeter, in unprotected places, such as squares, parks, and avenues. To determine the areas of a significant flow of people, the "traffic" tool on Google Maps® was used.

After determining points of interest, the locations were analyzed in loco regarding the trees available in the location, analyzing their condition and conservation. Older trees, with hollows, decaying material and cracks were included for sampling.

Figure 1-Spatial distribution of the locations of collected points (red dots)in the city of Birigui-SP



Fonte: Araújo and Marinho, (2023, p.04)

Sample Collection

There are colleted 100 samples from environment, from tree holes, bark, decomposing matter, and fragments of trunks, using sterile curettes to scrape the material. The samples were stored for transport in sterile universal collector-type flasks with a

screw cap, for further processing.The identification of the family, genus, and species of the trees included in the survey was previously carried out using the mobile applications PictureThis© and PlantNet©, and later at the Environmental department of Araçatuba.

Samples processing

The samples were processed in a laminar flow hood, where the collected material was homogenized and a 1g aliquot separated, which was macerated using a sterile mortar and pestle. After this process, the macerate was transferred to an Erlenmeyer flask with 50 mL of sterile 0.9% saline solution with chloramphenicol (400 µg/mL). The preparation was sealed and vigorously stirred using a vortex for five minutes, after which it was allowed to rest for 30 minutes at room temperature. After this time, 10 mL of the supernatant was transferred to tubes and centrifuged at 1500 RPM for five minutes. After centrifugation, nine mL of the supernatant was discarded, and the remaining volume was homogenized with the sediment.

Sample Cultivation

The homogenized volume (100µL) were seeded with Drigalski strap in a Petri dish containing Niger Seed Agar medium (NSA) (RANDHAWA *et al*.,2005), then draining the content to two more plates with the same medium. The same procedure was performed, with a flamed loop, in tubes containing Sabouraud Dextrose Agar (SDA) with chloramphenicol. All inoculated media were incubated for 5 days at a temperature of 25ºC (FIOCRUZ,2015). Colonies with creamy textures, with beige or brown pigmentation, were spread on NSA plates until pure colonies were obtained. Subsequently, these were spiked in SDA for identification and maintenance of the sample. All samples were stained with Nankin ink and lactophenol-blue-cotton for presumptive identification of the genus *Cryptococcus*.

Phenotypic identification

Characteristic colonies for *Cryptococcus* spp. were seeded in tubes containing urea-agar base (CHRISTENSEN, 2013), incubated at 25ºC, being observed daily for 5 days. For the thermotolerance test, the samples were seeded in tubes containing SDA and incubated at 37ºC for seven days (STEENBERGEN and CASADEVALL, 2003; ANVISA, 2013) analyzing their development daily.Positive samples for the urease and thermotolerance tests were sown on CGB (canavanine-glycine-bromothymol blue)

médium (KWON-CHUNG *et al.*,1982) for presumptive differentiation of the *C. neoformans and C. gattii* species complexes. The isolates were picked and incubated at 25°C for up to five days, and the color of the medium was observed daily. Strains WM148 (*C. neoformans* VNI) and WM178 (*C. gattii* VGII) were used as positive controls of the medium.

Carbon and nitrogen assimilation tests were also carried out, using specific solid media (LACAZ, 2002). For the carbon assimilation test, glucose (positive control), sucrose, lactose, galactose, raffinose, inositol, xylose, cellobiose, trehalose, dulcitol, maltose, and melibiose were used, and as a source of nitrogen, potassium nitrate and bacterial peptone (positive control) were used. Tables by Sidrim (2004) and Fonseca, Boekhout, and Fell (2011) adapted to the study were used as a comparison standard. Strains WM148 (*C. neoformans* VNI), WM626 (*C. neoformans* VNII), WM178 (*C. gattii* VGII), and *C. laurentii* (positive strain provided by the Adolfo Lutz Institute) were used as controls. All samples that presented morphophysiological and biochemical characteristics compatible with the of *C. laurentii, C. neoformans* and *C. gattii* species complexes continued for molecular characterization.

Molecular characterization

Samples were incubated in SDA for 48 hours at 25ºC and proceeded to DNA extraction and PCR-RFLP as described by Martins and coworkers (MARTINS *et al.*,2007) with some modifications. After growth, a portion of the colonies was transferred to a microtube containing 1mL of 50mM EDTA, which was centrifuged at 10,000 RPM for 8 minutes. The supernatant was discarded and the pellet was resuspended in 200µL of 50mM EDTA pH 8.0 with 40µL of a lytic enzyme from Trichoderma harzianum (60mg/mL), being subsequently homogenized by vortexing and incubated in a water bath for 30 minutes at 37ºC. After the incubation time, the sample was centrifuged for 8 minutes at 10,000 RPM and the supernatant was discarded. To the sediment was added 300µL of lysis buffer solution (10mM Tris-HCl pH 8.0, 10mM EDTA pH 8.0, 0.5% SDS, 0.01% N-laurylsarcozyl and 110µg/mL proteinase K), homogenized in vortex and incubated at 56ºC for 30 minutes in a water bath. After the time elapsed, the sample was centrifuged for 8 minutes at 10,000 RPM and the supernatant was transferred to another microtube. 300µL of chloroform/isopropanol (24:1) solution was added to the supernatant, which was manually shaken and centrifuged for 8 minutes at 10,000 RPM.

The aqueous supernatant was pipetted into another microtube, 400µL of isopropanol was added and centrifuged for 8 minutes at 10,000 RPM. After this step, the supernatant was discarded, 300µL of 70% ethanol was added and centrifuged for 8 minutes at 10,000 RPM. Finally, the supernatant was discarded and the microtube with DNA was left in the overnight flow hood for complete evaporation of the 70% ethanol. DNA was eluted in 20µL of H2O for PCR.

After elution, the DNA was quantified and analyzed for its purity, and the concentration of the samples was adjusted to 50 ng/µL. After standardizing the samples, two specific primers for the complexes of *C. neoformans* and *C. gattii* species complexes were used, with the sequences: URA5 (5' ATG TCC TCC CAA GCC CTC GAC TCC G 3') and SJ01 (5' TTA AGA CCT CTG AAC ACC GTA CTC 3'). For the PCR reaction, was used 1µL of sample or control, 24µL of the Mastermix preparation - GoTaq® Green Master Mix (Promega), primers (25 pM) URA5 and SJ101 and sterile distilled water. The thermocycling profile of the samples consisted of 37 cycles: one cycle of 94°C for 5 minutes, 35 cycles consisting of 3 steps (94°C for 45 seconds, 63°C for 60 seconds, and 72°C for 1 minute), and a final extension at 72°C for 10 minutes. Sample and control fragments were analyzed on a 2% agarose gel and visualized using an ultraviolet light transilluminator. After amplification of the fragments, the PCR products were digested twice by the restriction enzymes Sau96I and HhaI at 37ºC for 3 hours to determine molecular types, and a new visualization of bands was performed on a 2% agarose gel. The standards of comparison were the control samples WM148 (VNI/AFLP1), WM626 (VNII/AFLP1A), WM628 (VNIII/AFLP2), WM629 (VNIV/AFLP3), WM179 (VGI/AFLP4), WM178 (VGII/AFLP6), WM175 (VGIII/AFLP5), WM779 (VGIV/AFLP7)(3) and *C. laurentii* (IAL).

Statistical tests and mapping

Variables were analyzed using the Chi-square test and descriptive statistics for collected and positive samples for tree species and family, seasons, and origin of the collected material were used. For the association of results regarding the complex of species/species, trees, and origin of the collected material, the Kruskal-Wallis test was used. A significance level of 5% ($p < 0.05$) was adopted in both tests, graphs and calculations were performed using the GraphPad Prism 9© program. The maps were

created using the QGIS 3.20 software, Datum SIRGAS 2000 geographic coordinate system, IBGE 2019 and Waze 2023© cartographic base.

RESULTS

From February 7, 2018 to March 9, 2019 a total of 100 samples were taken at various points in the city of Birigui. The results showed, six *C. laurentii* samples, five *C. gattii* VGII samples, and 1 *C. neoformans* VNIV sample were obtained using molecular means, and three *C. albidus* samples using assimilation of carbon and nitrogen compounds (Table 1). The carbon assimilation test for *C. gattii* VGII and *C. neoformans* VNIV samples assimilated different sources than indicated in the comparison table. The *C. laurentii* samples maintained the described profile, except samples A33.N3.1, A50.N1.1, A61.N2.2, and A81.N2.1 which showed a weak assimilation of potassium nitrate. Samples A35.N1.1, A38.N1.7 and A91.N2.2 were characterized as *C. albidus* by the profile shown in the carbon and nitrate assimilation test.

Table 1 – Isolated samples of *C. neoformans* and *C. gattii* molecular types, *C. laurentii* and *C. albidus* species; tree species and family.

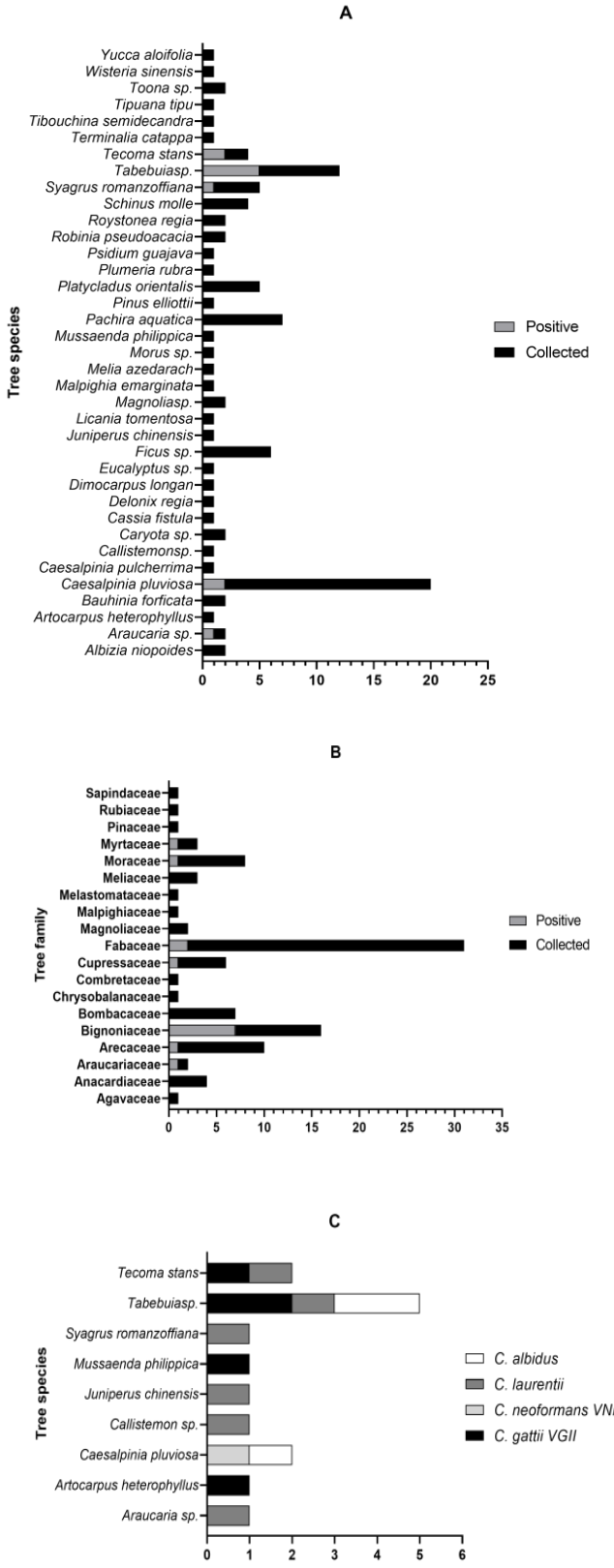
Sample	Species/Molecular Type	Station	Tree name	Tree family
A14.N1.2	*C. gattii* VGII	Autumn	*Tecoma stans*	Bignoniaceae
A24.S2.2	*C. neoformans* VNIV	Autumn	*Caesalpinia pluviosa*	Fabaceae
A33.N1.1	*C. laurentii*	Autumn	*Tecoma stans*	Bignoniaceae
A35.N1.1	*C. albidus*	Autumn	*Tabebuia* sp.	Bignoniaceae
A35.N1.2	*C. gattii* VGII	Autumn	*Tabebuia* sp.	Bignoniaceae
A38.N1.7	*C. albidus*	Autumn	*Tabebuia* sp.	Bignoniaceae
A42.N1.1	*C. laurentii*	Winter	*Syagrus romanzoffiana*	Arecaceae
A48.N3.4	*C. gattii* VGII	Winter	*Artocarpus heterophyllus*	Moraceae
A50.N1.1	*C. laurentii*	Winter	*Callistemon* sp.	Myrtaceae
A54.N3.1	*C. laurentii*	Winter	*Araucaria* sp.	Araucariaceae
A61.N2.2	*C. laurentii*	Winter	*Tabebuia* sp.	Bignoniaceae
A61.S3.1	*C. gattii* VGII	Winter	*Tabebuia* sp.	Bignoniaceae
A81.N2.1	*C. laurentii*	Summer	*Juniperus chinensis*	Cupressaceae
A83.S3.3	*C. gattii* VGII	Summer	*Mussaenda philippica*	Rubiaceae
A91.N2.2	*C. albidus*	Summer	*Caesalpinia pluviosa*	Fabaceae

Fonte: Araújo and Marinho (2023, p.08)

The association between the results expressed for the species of *Cryptococcus* and the total number of samples collected per family of trees showed no difference significant (p=0.14), however, when analyzed only among the families of trees with positive isolations for *Cryptococcus* species, there was a significant difference (p=0.026), with the Bignoniaceae family having 46.6% of the total of positive isolations. There was also no statistical difference when analyzing the total number of tree species collected by the total number of isolations, and only between tree species with positive isolations (p=0.14 and

p=0.065 respectively), nor a predilection between the species analyzed by one family (p=0.26) or specific species (p=0.12) (Figure 2).

Figure 2 - A – Association between the results of the total number of samples taken by the total number of tree species; B – Association between the total number of samples collected and the total number of isolates per tree family; C – Association between *C. neoformans* and *C. gattii* molecular types, *C. laurentii* and *C. albidus* species by tree species and families

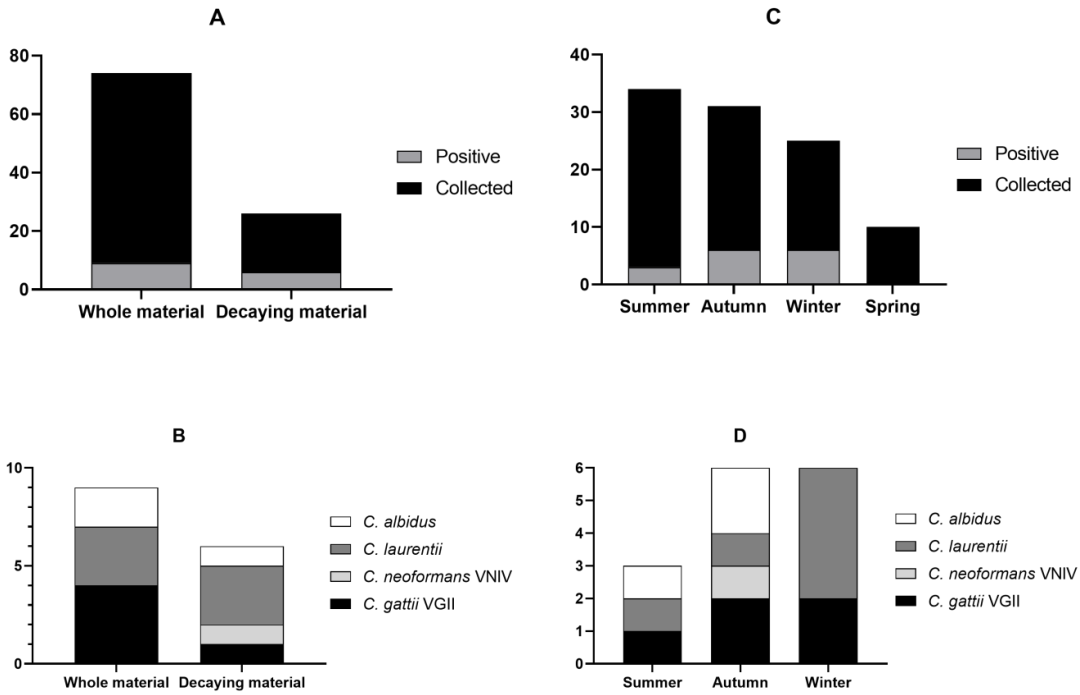


Fonte

Fonte: Araújo and Marinho (2023, p.09)

As for the seasons, the comparison between the total number of isolates and the season (p=0.184), it was found that most of the positive samples for *Cryptococcus* species were isolated during winter and autumn (80% of the total positives), with a temperature below 20°C. Regarding the association between the origin of the material collected and the total amount of isolates, there was no statistical difference between the analyzed data (p=0.30), as well as the predilection for *Cryptococcus* species by material (p=0.31) or season (p=0.25) specifically, as shown in Figure 3.

Figure 3 A – Association between the total number of collected samples and isolations by material source; B – Association between *C. gattii* and *C. neoformans* species complexes isolates and *C. laurentii* and *C. albidus* species by origin of the material; C – Association between the total number of samples collected and the total number of isolations per season; D – Association between the isolations of the *C. gattii* and *C. neoformans* species complexes isolates and *C. laurentii* and *C. albidus* species by climatic season.



Fonte: Araújo and Marinho (2023, p.10)

The spatial distribution of isolations can be seen in Figure 4. Note that 4 positive samples were isolated in Parque do Povo, with two different species being isolated from sample A35 (*C. gattii* VGII and *C. albidus*), 2 in Capela São João Batista and 2 isolates from sample A61 (*C. gattii* VGII and *C. laurentii*) from Praça Beatriz Calixto Sanches. The other positive samples (n=7) were unique samples for their location.

Figura 4 Spatial distribution of collected samples and species/species complexes, identified according to their characterization

Fonte: Araújo and Marinho (2023, p.11)

DISCUSSION

The results of this research demonstrated a slightly higher amount of positive isolates for the number of environmental samples collected, generally around 10%, as reported in the literature (ANZAI *et al*.,2014; GERSTEIN *et al*.,2017), but it is similar to the data expressed by Araújo-Júnior *et al*., (2015), and researchers in Araçatuba, although they are not endemic cities for cases of cryptococcosis, as observed in the north and northeast of Brazil (DAMASCENO-ESCOURA *et al*.,2019). It is worth mentioning that the isolation of pathogenic species of the genus *Cryptococcus* from the environment is usually difficult, given the number of microorganisms that are part of the natural microbiota of trees, mainly fast-growing filamentous fungi, not favoring the visualization of yeast colonies, which can lead to false-negative results (FORTES *et al*,2001; MAK *et al.*, 2015). Regarding the species of trees collected, the results presented corroborate with other works, attesting that the *C. gattii* and *C. neoformans* species complexes are not directly associated with a specific species of trees (HAGEN and BOEKHOUT,2010), but with the natural biodegradation of organic compounds and wood (FORTES *et al*,2001). However, each species complexes behaves differently, with the *C. neoformans* species complexes being more associated with the bird, bat, and soil excreta (COLOMBO *et al.*, 2015; VIEILLE *et al*.,2018), unlike the *C. gattii* species complexes that is present in dust, leaves, fruits, plants and insects (ACHESON *et al*., 2018), and the species *C. laurentii* in vegetables, milk and also in bird excreta (GUPTA *et al*.,2018). Another important

consideration is that the locations of the isolates configure a micro-niche, indicating that the positive isolates tend to appear close to each other. It is suggested that geographic differences are relevant in terms of their dispersion and maintenance in the environment (ROMEO *et al*.,2012), but the low amount of environmental isolation about clinical isolations and underreporting of cases hinder studies on the expansion and distribution of these species in the environment (MONTAGMA *et al*.,2018; SUN *et al*.,2019). The climate and the season of the year were also relevant factors, although there are few climatological studies together with the study of *Cryptococcus* isolation, those close to 0ºC of the Canadian winter (ACHESON *et al*.,2018). However, it is recognized that the different molecular types have different climatic requirements in nature to favor their maintenance in the environment (GRAMADOS and CASTANEDA 2006), these variations may even interfere with their environmental isolation (GERSTEIN and NIELSEN,2017). There are also changes observed in recent years, such as global warming and El Niño, factors that may be directly linked to the appearance of outbreaks in countries where cryptococcosis was previously rare (ACHESON *et al.*,2018). Some strains cataloged in Brazil are considered more genetically conserved, suggesting that the Amazon is the cradle of the pathogenic species of the genus (HAGEN *et al*.,2013). Other authors found great genetic diversity in their isolates in several states of the country. The distributions of molecular types in Brazil are heterogeneous (TRILLLES *et al*.,2008), unlike the profiles found in other parts of the world, where there is only less diversity of species (MEYER and TRILLES,2010), possibly due to the continental size of the country. In Brazil, the most prevalent molecular types in cryptococcosis are VNI for immunosuppressed individuals and VGII for immunocompetent individuals (DAMASCENO-ESCOURA *et al*.,2019). *C. gattii* VGII has been linked to outbreaks on Vancouver Island, considered one of the most virulent types among the species complexes (13), which is the only molecular type of *C. gattii* found in this work, in the city of Birigui. The VNIV molecular type is one of the least studied, as there are few environmental and clinical isolates, its prevalence is more linked to the European continent, with few isolates in Brazil (ANDRADE-SILVA *et al.*,2018). Cases of cryptococcosis by other species, such as *C. laurentii* and *C. albidus*, are rarely diagnosed and the lack of standardization in diagnoses and treatments means that there are few case reports in the literature (GUPTA *et al*.,2018). The results revealed that *C. albidus* strains have a biochemical profile similar to *C. neoformans* and *C. laurentii* to *C. gattii*, being only identified and

characterized with the association of tests of assimilation of sugars, nitrogen and PCR. Recently, cases of hybrid strains have been reported, with changes in the patterns of the URA5 gene fragments due to mutations (FOREK *et al*.,2019). The pattern found in our samples of *C. laurentii* showed an identical RFLP profile to those reported by Andrade-Silva et al. (2010), being these the only studies found that used the PCR-RFLP technique with the URA5 primer and double digestion of enzymes for this species. Unlike the behavior expressed by *C. albidus*, which did not show results with the URA5 primer, being identified by traditional tests, such as the assimilation of sugars and nitrogen. The use of molecular techniques greatly contributes to the identification of strains, for a better understanding of eco-epidemiology, pathogenicity and diversity of species, as well as the genetic variability that make up the genus *Cryptococcus*. However, due to the great diversity of species, the association between molecular and traditional techniques is of paramount importance in the correct identification of the species, especially when dealing with clinical samples (COSTA,2008). It is noteworthy that, in the past, the *C. gattii* species complex was also underreported when compared to *C. neoformans*, until it was considered an important pathogen worldwide, as recently, with the increase in reports of cases of cryptococcosis for this species.

CONCLUSION

In this study, the mapping of tree environmental samples belonging to the *C. gattii* and *C. neoformans* species complexes, as well as the *C. laurentii* and *C. albidus* species, from the city of Birigui. There was no association between the factors of tree species, the season of the year, and the origin of the collected material to a certain species of *Cryptococcus*. Due to the complexity and diversity of the species that make up the genus, the association between traditional and molecular techniques is of vital importance for the identification and characterization of these species.

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