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AVALIAÇÃO CLÍNICA E ECOEPIDEMIOLÓGICA DE LEVEDURAS DE INTERESSE MÉDICO

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Avaliação Clínica e Ecoepidemiológica de Leveduras de Interesse Médico

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SUMÁRIO

DEDICATÓRIA

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EPÍGRAFE

"Existe apenas um bem, o saber,
E apenas um mal, a ignorância".
Sócrates

LISTA DE FIGURAS

Artigo 1

LISTA DE TABELAS E QUADROS

Artigo 1

Artigo 2

Artigo 3

LISTA DE ABREVIATURAS E SÍMBOLOS

5-FC – 5-fluorocitosina

AFLP - Amplified Fragmet Length Polymorphism Analysis

AIDS - Acquired Immunodeficiency Syndrome

ATCC 90012 - American Type Culture Collection

CHEF - Contour-clamped Homogeneous Electrophoresis

CLSI - Clinical and Laboratory Standards Institute

CSF - Cerebrospinal fluid

DFIs – Doenças Invasivas Fúngicas

DNA – Desoxiribonucleic acid

EDTA - Ethylenediamine tetraacetic acid

EUCAST - European Committee on Antimicrobial Susceptibility Testing

FAMERP – Faculdade de Medicina de São José do Rio Preto

HIV – Human Imunodeficiency Virus

MDR - multidrug-resistant

MICs - Minimum Inhibitory Concentrations

MLP - Microsatellite Length Polymorphism

MLST - Multilocus Sequence Typing

NSA - Niger seed agar

PFGE - Pulsed-field Gel Electrophoresis

RAPD - Randomly Amplified Polymorphic DNA

RFLP – Restriction Fragment length Polymorphism

S
AB – coeficiente de similaridade

SNC – Sistema Nervoso Central

SSCP - Single-Strand Conformation Polymorphism Analysis

TBE – Tris-HCl, boric acid, EDTA

UNESP – Universidade Estadual Paulista

UNIFEV – Centro Universitário de Votuporanga

UPGMA - Unweighted Pair Group Method with Arithmetic mean

USA – United States of America

VVC - Vulvovaginal candidiasis

RESUMO

Introdução: A incidência das doenças fúngicas invasivas têm aumentado mundialmente tendo como principais patógenos o *Cryptococcus* spp e *Candida* spp. Vários fungos capazes de ocasionar patologias humanas são espécies ambientais, cuja patogenicidade evoluiu a partir das estratégias desenvolvidas no ambiente de estresse. **Objetivos:** Realizar uma avaliação clínica e ecoepidemiológica de isolados de leveduras de interesse médico. **Material e Método:** Amostras clínicas de líquor obtidas de pacientes portadores do vírus HIV com meningite foram analisadas quanto a presença de *Cryptococcus* spp. Os isolados foram identificados pelas características bioquímicas, com posterior análise genética pela técnica de RAPD para comparação filogenética. O perfil de suscetibilidade aos antifúngicos (fluconazol, itraconazol, anfotericina-B e 5-fluorocitosina) foi avaliado para todas as amostras, utilizando a técnica de microdiluição. Investigação ambiental em árvores e fezes de frangos de corte foi realizada para determinar a presença de leveduras de interesse médico, com possível correlação filogenética às amostras clínicas. Essas amostras também foram avaliadas quanto ao perfil de suscetibilidade aos antifúngicos, entretanto com a técnica de difusão em disco. **Resultados:** Quarenta isolados de *Cryptococcus* spp foram obtidos de 35 pacientes, sendo 39 identificados como *C. neoformans* e 1 com *C. gattii*. Cinco pacientes apresentaram isolados sequenciais (cada um com dois isolados). Trinta e seis (90%) pertenciam ao sorotipo A, 7,5% ao sorotipo AD e 2,5% ao sorotipo B. A análise de RAPD mostrou 6 grupos com 13 subgrupos, com elevado coeficiente de similaridade. A maioria dos isolados foi considerada sensível aos antifúngicos testados. Três isolados clínicos apresentaram colônias com produção tardia da enzima fenoloxidase, sendo a essas amostras acrescentadas as análises de quantificação da enzima fenoloxidase e análise genética utilizando enzimas de restrição e eletroforese. Não foi encontrada alteração no padrão genético com características fenotípicas nessas amostras. Quatrocentas amostras de material em decomposição de árvores foram analisadas no período de 2015 a 2016, com isolamento de *C. tropicalis*, *C. albicans*, *C. krusei*, *C. glabrata*, *C. parapsilosis*, *C. guilliermondii* e *C. laurentii*. Elevado percentual das amostras demonstrou resistência aos azóis. As amostras de fezes de frango de corte totalizaram 180, com maior porcentagem de isolamento de espécies não-*albicans*. Entretanto, as mesmas apresentaram elevado perfil de sensibilidade aos antifúngicos. Não

houve isolamento de *Cryptococcus* spp nas amostras ambientais, não sendo possível realizar uma correlação filogenética com as amostras ambientais. **Conclusão:** Priorizar estudos ecoepidemiológicos são necessários para compreender o ecossistema que os fungos patogênicos estão inseridos, contribuindo na compreensão genética-fenotípica na patogênese. Fato esse essencial para implementação de técnicas diagnósticas e terapêuticas, assim como medidas preventivas com ênfase na prevenção de doenças na comunidade.

ABSTRACT

Introduction: The incidence of invasive fungal diseases has increased worldwide with *Cryptococcus* spp and *Candida* spp. Several fungi capable of causing human pathologies are environmental species whose pathogenicity evolved from the strategies developed in the stress environment. **Objectives:** To carry out a clinical and ecoepidemiological evaluation of yeast isolates of medical interest. **Material and Method**: Clinical samples of CSF obtained from patients with HIV were analyzed for the presence of *Cryptococcus* spp. The isolates were identified by biochemical characteristics, with subsequent genetic analysis by the RAPD technique for phylogenetic comparison. The antifungal susceptibility profile (fluconazole, itraconazole, amphotericin-B and 5-fluorocytosine) was evaluated for all samples using the microdilution technique. Environmental investigation in trees and feces of broilers was performed to determine the presence of yeasts of medical interest, with possible phylogenetic correlation to the clinical samples. These samples were also evaluated for the antifungal susceptibility profile, however with the disc diffusion technique. **Results:** Forty *Cryptococcus* spp isolates were obtained from 35 patients, 39 of whom were identified as *C. neoformans* and 1 with *C. gattii*. Five patients had sequential isolates (each with two isolates). Thirty-six (90%) belonged to serotype A, 7.5% to AD serotype and 2.5% to serotype B. RAPD analysis showed 6 groups with 13 subgroups, with a high coefficient of similarity. Most isolates were considered sensitive to the antifungal agents tested. Three clinical isolates presented colonies with late production of the enzyme phenoloxidase, adding to these samples the quantification analysis of the enzyme phenoloxidase and genetic analysis using restriction enzymes and electrophoresis. No change was found in the genetic pattern with phenotypic characteristics in these samples. Four hundred samples of decomposing material were analyzed in the period from 2015 to 2016, with isolation of *C. tropicalis*, *C. albicans*, *C. krusei*, *C. glabrata*, *C. parapsilosis*, *C. guilliermondii* and *C. laurentii*. High percentage of the samples showed resistance to azoles. Samples of feces of broiler chicken totaled 180, with a higher percentage of isolation of non-albicans species. However, they had a high antifungal sensitivity profile. There was no isolation of *Cryptococcus* spp in the environmental samples, and it was not possible to perform a phylogenetic correlation with the environmental samples. **Conclusion:** Prioritizing ecoepidemiological studies are

necessary to understand the ecosystem that the pathogenic fungi are inserted, contributing in the genetic-phenotypic understanding in the pathogenesis. This fact is essential for the implementation of diagnostic and therapeutic techniques, as well as preventive measures with an emphasis on disease prevention in the community.

INTRODUÇÃO

Nas últimas décadas, tem ocorrido um aumento na incidência mundial de patologias fúngicas e disseminação de fungos emergentes. Esses eventos podem estar associados a vários fatores, incluindo aos relacionados às defesas naturais do hospedeiro, tais como: os portadores do vírus da imunodeficiência humana (HIV), os transplantados, os oncológicos, sob terapia imunossupressora, os prematuros e os idosos; aos deslocamentos migratórios humanos e aos fatores ambientais, tais como aquecimento global que contribuem para pressão seletiva no desenvolvimento de fatores de virulência. (1-10)

Os fungos compreendem um vasto e diversificado grupo de microrganismos, mas somente algumas espécies são consideradas patógenos humanos. A maioria é parasita facultativo, e infectar o hospedeiro não é uma parte obrigatória do ciclo da vida. A temperatura corpórea do hospedeiro é uma barreira protetiva contra várias espécies que se desenvolvem melhor a temperatura ambiente. Vários fungos capazes de ocasionar uma micose sistêmica em humanos são espécies ambientais, cuja patogenicidade evoluiu a partir das estratégias desenvolvidas no ambiente de estresse.(11,12)

As doenças fúngicas invasivas (DFIs) estão associadas a elevada morbidade e mortalidade, tendo como os patógenos fúngicos mais comuns: *Candida, Aspergillus*, *Cryptococcus* e *Pneumocystis* spp; ocasionando mais de 90% de óbitos entre as patologias fúngicas. Entretanto, as doenças fúngicas são negligenciadas mundialmente, reflexo da dificuldade de diagnóstico devido à manifestação de sintomas não específicos e a falta de programas vigilância epidemiológica que dificultam as estimativas e contribuem para dados subestimados.(1,5,11,13-15)

A criptococose afeta aproximadamente 1milhão de pessoas no mundo, com estimativa de ocasionar mais de 600.000 mortes/ano. Na América Latina, mais de 5.000 novos casos de meningite criptocócica são notificados com 2.400 óbitos. O Brasil é o país de maior incidência (1.000-2.500 casos), com taxas de mortalidade variando entre 32-60%.[16-23]

A criptococose é ocasionada pela levedura capsulada *Cryptococcus*, pertencente ao filo Basidiomiceto. A infecção pulmonar primária é decorrente da inalação de propágulos infectantes da levedura presentes na natureza, principalmente em fezes secas de aves e restos de madeira em decomposição, dispersos pelo ar. A disseminação para outros sítios anatômicos ocorre via hematogênica, com tropismo ao Sistema Nervoso Central (SNC) ocasionando a meningoencefalite criptocócica subaguda ou crônica, a manifestação mais comum e fatal na ausência de tratamento. Infecção renal, hepática, óssea, cutânea, próstata, sanguínea e ocular têm sido documentadas.[28-37]

Até recentemente, o *Cryptococcus* spp tinha sido classificado em duas espécies com cinco sorotipos (A-D). Várias técnicas moleculares têm sido amplamente usadas para elucidar questões acerca da epidemiologia, do tratamento e da patogênese, revelando diversidade genética significante, ocasionando controversa na nomenclatura. As espécies do *Cryptococcus* podem ser classificadas em dois complexos de maior relevância: espécies do complexo *C. neoformans* (*C. neoformans* e *C.* deneoformans), tipos moleculares VNI, VNII, VNIII e VNIV e espécies do complexo *C. gattii* (*C. gattii*, *C. deuterogattii*, *C. bacillisporus*, *C. tetragattii* e *C. decagattii*), tipos moleculares VGI, VGII, VGIII e VGIV. O *C. neoformans* tem distribuição mundial e o *C. gattii* é comumente encontrado em regiões tropicais e subtropicais; entretanto, essa espécie foi

evidenciada no Canadá (Ilha Vancouver, Columbia Britânica) e nos Estados Unidos (Noroeste do Pacífico).[38-40]

Mundialmente, *C. neoformans* (VNI) causa mais de 90% dos casos de criptococose na América Latina. Já, o *C. gattii* (VGII e VGIII) afeta indivíduos saudáveis. Os tipos moleculares VNI e VGII são mais frequentes no Brasil, corroborando com dados relatados na Argentina, Chile, Colombia, Cuba, Equador, Guatemala, Honduras, Mexico, Paraguai, Peru, Uruguai e Venezuela.[24,33,36,39,40-48]

Os fatores de virulência das espécies do complexo *Cryptococcus* incluem cápsula polissacarídica, melanina, habilidade de crescer na temperatura corpórea (37°C), fosfolipase B, urease, entre outros. Isolados obtidos do meio ambiente apresentam menor virulência quando comparados com isolados clínicos, sugerindo variação genética natural. A produção de melanina é o principal fator de virulência, mas pouco se conhece sobre variações na quantificaçao e fatores genéticos. Compreender a pressão seletiva se faz necessário para elucidar a correlação da diversidade genética com a virulência em humanos.[49-52]

Independente das manifestações clínicas, o tratamento precoce da meningite criptocócica minimiza o risco de mortalidade. O recomendado se faz pela combinação de anfotericina-B, seguido de azóis (cetoconazol, itraconazol, fluconzaol e voriconazol) para consolidação, terapia de manutenção e ocasionalmente na profilaxia. A microevolução pode ocorrer em virtude da pressão seletiva das drogas, levando a resistência causando infecções persistentes.[53-56]

O número de pacientes imunocomprometidos suscetíveis as DFIs tem crescido, aumentando o uso de antifúngicos sistêmicos para tratamento e profilaxia, minimizando o risco de mortalidade. Em contrapartida, a ampla exposição aos antifúngicos potencializa a

emergência de resistência contribuindo com o avanço das DFIs em pacientes de alto risco. (57-58)

A infecção da corrente sanguínea ocasionada por *Candida* spp é a mais frequente em pacientes hospitalizados, com taxa de mortalidade excedendo os 40% no Brasil. A fonte endógena de contaminação é a mais frequente, uma vez que essa levedura é considerada comensal em humanos podendo ser encontrada na microbiota oral, gastrointestinal, genital e pele.(15,59-68)

A *Candida albicans* é responsável pela maioria dos casos de candidemia; entretanto, estudos epidemiológicos recentes têm demonstrado um decréscimo da frequência dessa e um aumento da prevalência das espécies não-*albicans*. Dentre as espécies não-*albicans* destacam-se: *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. lusitaniae*, *C. guilliermondii C. famata*. A *Candida auris* está associada com elevadas taxas de mortalidade desde 2008, ano descrita pela primeira vez como patógeno humano. (15, 67,68-79)

Testes de suscetibilidade aos antifúngicos de *Candida* spp e *Cryptococcus* spp são de extrema relevância para monitorar a ocorrência de cepas resistentes e otimizar estratégias de tratamento e prevenção das DFIs. Sendo assim, se faz necessária a utilização de técnicas *in vitro* padronizadas para que os resultados obtidos tenham correlação com o sucesso terapêutico. A microdiluição, E-test e difusão em disco são métodos comumente utilizados, demonstrando variedade no perfil de suscetibilidade das leveduras relatadas. (65,80,81,82)

A resistência de *Candida* spp ao fluconazol tem sido amplamente demonstrada, não somente pela *C glabrata* mas também por *C. parapsilosis* e *C. tropicalis*, inicialmente descritas com sensibilidade primária aos azóis. Esse padrão tem sido relatado em outros

estudos na América Latina, com diversidade nas taxas de resistência nas regiões do Brasil. Acrescido a esse cenário, o amplo uso de equinocandinas em terapia profilática e empírica tem mudado a realidade com crescente número de *Candida* não-*albicans* resistente a múltiplas drogas.[67,69,70,71,77,82-92]

Impacto semelhante tem sido descrito com *Cryptococcus* spp. com sensibilidade reduzida aos azóis, mais especificamente ao fluconazol. No Brasil, estudos de suscetibilidade *in vitro* tem demonstrado percentual elevado de isolados sensíveis à anfotericina-B e 5-fluorocitosina, estando em desacordo algumas regiões. Dados semelhantes aos encontrados no Brasil foram mostrados em estudos na América Latina. [6,36,44,93-97]

Nichos ecológicos de *Cryptococcus* spp e *Candida* spp têm sido extensivamente estudados, quebrando paradigmas sobre a distribuição geográfica dos patógenos. Adaptações às diferentes condições climáticas e ambientais são cruciais na evolução das espécies, influenciando a concentração dessas leveduras no solo, árvores, água, ar e fezes de animais e consequentemente aumentando o tempo de exposição ao hospedeiro. A tolerância aos estressores ambientais bióticos e abióticos, naturais ou artificiais, podem ser vantajosos para o microrganismo no meio ambiente. Dados científicos comprovaram o envolvimento da termotolerância e resistência aos agrotóxicos com outros fatores de virulência, atributos essenciais para determinar a patogenicidade das leveduras, destacando a interação entre fatores genéticos e ambientais que levam à variação fenotípica.[4,39,49,98-131]

Vários métodos moleculares desenvolvidos na década de 1980 foram abandonados pela dificuldade de desempenho ou resultados inadequados para a genotipagem, tais como: PFGE (pulsed-field gel electrophoresis), RFLP (Restriction Fragment length

Polymorphism), RAPD (Randomly Amplified Polymorphic DNA), SSCP (Single-Strand Conformation Polymorphism Analysis), AFLP (Amplified Fragmet Length Polymorphism Analysis), MLP (Microsatellite Length Polymorphism), MLST (Multilocus Sequence Typing). Ainda assim, o RAPD tem sido extensivamente usado para estudos epidemiológicos de vários fungos patogênicos.[120,132-140]

Análise filogenética de isolados ambientais de *Cryptococcus* spp do Brasil sugerem migração de linhagens entre África e américa do Sul, demonstrando uma distribuição multi-continental devido a elevada dispersão natural desse patógeno. Comparação filogenética entre isolados ambientais e clínicos representam a base para abordar futuras investigações e intervenções de saúde pública.[48,100,107,110, 112,141,142]

Estudos eco-epidemiológicos são relevantes para elucidar a ecologia e etiologia das fungos patogênicos. Compreender a emergência e evolução contínua do patógeno em um novo reservatório ambiental é crucial para a epidemiologia das doenças, fundamentais para priorizar estratégias de prevenção, diagnósticas e de intervenções terapêuticas, essenciais para a promoção da sáude coletiva.

OBJETIVO

A presente pesquisa objetivou avaliar a presença de leveduras de interesse médico em amostras ambientais e o perfil de suscetibilidade aos antifúngicos.

Como objetivos secundários pretendeu-se correlacionar filogeneticamente amostras clínicas e ambientais de *Cryptococcus* spp e avaliar o perfil de suscetibilidade aos antifúngicos.

ARTIGO CIENTÍFICO 1

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HIGH GENETIC SIMILARITY BETWEEN CLINICAL SAMPLES OF *C. neoformans* FROM DIFFERENT REGIONS OF SÃO PAULO STATE

GENETIC CORRELATION IN *C. neoformans*

ABSTRACT

Introduction: Cyptococcosis is a systemic fungal disease with high lethality. Genotyping of infectious agents can understand the geographical distribution, defined reinfection or relapse and correlate with phenotype. In this way, this study sought to understand the phylogenetic relationship between *Cryptococcus* isolates and correlate with antifungal sensitivity profile and phenoloxidase activity. Methods: Forth isolates of *Cryptococcus* sp were collected of 35 HIV patients form different regions of São Paulo State. All isolates were analysed by the RAPD and microdilution technique. Three samples presented alteration in the production of the enzyme phenoloxidase, which was quantified and evaluated by Electrophoretic Karyotyping. Results: From a total of 40 cryptococcal isolates, 39 were identified as *Cryptococcus neoformans,* only one was *C. gattii*. The 5 patients with sequential isolates (each of them had 2 isolates) were identified *C. neoformans,* on both isolates. Thirty six (90%) isolates belong to serotype A, three to serotype AD (7,5%) and one to serotype B (2.5%). RAPD analyses showed 6 clusters with 13 subgroups, most of them with high coefficient of similarity. In our study, most of the isolates were considered susceptible with dose-dependence to the drugs fluconazole and itraconazole. Of the three patients who presented serial samples, one showed different genetic profiles and two, the same genetic profile in both samples, without altering the susceptibility profile. Conclusion: Studies to evaluate the relationship between molecular profile and phenotypic characteristic, such as virulence factors, resistance of antifungal

drugs are necessary to search for new molecular markers for genotyping, pathogenicity and drug susceptibility.

Key-words: *Cryptococcus*, susceptibility, genotyping, phenoloxidase, RAPD.

INTRODUCTION

Cryptococcosis is a systemic fungal disease associated with high lethality. The ubiquitous encapsulated yeast, *Cryptococcus* belonging to the phylum of basidiomycete, causes severe infection considered a systemic opportunist mycosis, particularly in patients with Acquired Immunodeficiency Syndrome (AIDS), hematological malignancies and therapeutic immunesuppression. However, it can also affect, less frequently, immunocompetent individuals [1-8].

Cryptococcosis still affects approximately 1 million people worldwide. In Latin America, this mycosis has significant morbid-mortality, with more than 5,000 new cases of cryptococcal meningitis each year, and 2,400 deaths[9]. Brazil was the country with high incidence (1,000-2,500 cases) with mortality rate of cryptococcal infections was 0.47 million inhabitants, the thirteenth cause of death[10-12].

The recommended treatment por patients with cryptococcal meningitis is a combination of amphotericin B, used for induction and flucytosine (5-FC), followed by fluconazole for consolidation or maintenance therapy and in occasional individual, prophylaxis. Microevolution can occur in response to drug pressure, leading to resistance causing relapse due to persisting infections. By the way, in the absence of continued anti fungal therapy in patients with HIV/AIDS also have a high probability of recurrence of meningitis cryptococcal [13-16].

Virulence factors for *Cryptococcus neoformans* species complex include a polysaccharide capsule, melanin, and the ability to grow at human body temperature (37^{o}C), phospholipase B, urease, and a number of signaling cascades. Isolates collected from the environment have been shown to be less virulent than those recovered from clinical sources, suggesting that there are natural genetic variation encoded within these could also affect virulence[17-21].

Several methods developed during the 1980s have now been practically abandoned by the difficult of perform or the results are not polymorphic enough for genotyping. Nonetheless, RAPD has been extensively used for studying epidemiology of several pathogenic fungi and it is still used[22-27].

Molecular methods suggest that different patients may be infected with the same genetic profile of Cryptococcus and among sequential isolates from de same patient, the profile of antifungal susceptibilty may be different. These could implicates with response to antifungal therapy, contributing with high rates of mortality[28-29]. In this way, this study sought to understand the phylogenetic relationship between Cryptococcus isolates and correlate with antifungal sensitivity profile and phenoloxidase activity.

METHODS

Fungal isolates

Forty isolates of *Cryptococcus* species were collected at different time intervals from cerebrospinal fluid (CSF) of 35 HIV patients from many different regions of São Paulo State. These isolates were maintained in the Culture Collection of the Instituto Adolfo Lutz Laboratory, a Public Health National Reference Center and of the Laboratory of Clinical Mycology of the Faculty of Pharmaceutical Sciences, UNESP - Araraquara.

Fourteen isolates originated from Araraquara, 3 from Jaboticabal and 23 from Ribeirão Preto. In the case of five of these patients, *Cryptococcus* isolates were recovered from two successive episodes of meningitis. the second sample was obtained during the period of maintenance therapy, performed with fluconazole. The remaining patients experienced a single episode of infection.

All isolates were identified to genus and species level based on micromorphological and biochemical characteristics. Following identification, isolates were subcultured to Sabouraud dextrose agar slants and frozen in 20% glycerol at –20^{o}C[30-32].

Antifungal susceptibility testing

I*n vitro* susceptibility of *C. neoformans* isolates to fluconazole (Pfeizer, USA), itraconazole (Janssen Pharmaceutical, USA), amphotericin-B (Sigma, USA), and flucytosine (5-FC) (Sigma, USA) was evaluated by broth microdilution method according to the Clinical and Laboratory Standards Institute—CLSI M27-A3 document. *Cryptococcus neoformans* ATCC 90012 was used as reference control organism. The tests were performed in duplicate. The minimum inhibitory concentrations (MICs) were determined by the lowest antifungal agent concentrations that inhibited 50% fungal growth compared to the control growth (without antifungal) for fluconazole, itraconazole and 5-FC; the MICs for amphotericin B were determined by the lowest concentrations that inhibited 100% growth[33].

RAPD analysis

Genomic DNA was extracted and purified by the method of Almeida et al. (2007).

The RAPD analysis was carried out with primer 6 (5'-d[CCCGTCAGCA]-3') from the

Ready-to-Go kit (Amersham Pharmacia Biotech) using a total volume of 25 μL, and in accordance with the manufacturer's instructions. The following PCR cycle conditions were used: initial denaturation at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 1 min, annealing at 36°C for 1 min and amplification at 72°C for 2 min, with a final extension at 72°C for 10 min. Amplification products were separated by electrophoresis on 2% agarose gels in 1X TBE buffer at 150 V for 2.5 h, stained with 0.5 $mg\ mL^{-1}$ ethidium bromide and visualized under UV light[29].

Banding patterns were analyzed by the ImageMaster VDS Software (Amersham Pharmacia Biotech). the genetic relationship of the isolates were determined by the computer program GelCompar II version 2.0. The similarity coefficient (S_{AB}) between patterns for every pair of isolates A and B was computed with the formula $S_{AB} = 2E / (2E+a+b)$, where E is the number of common bands in the patterns of A and B, a is the number of bands in pattern A with no correlates in pattern B, and b is the number of bands in pattern B with no correlates in pattern A. Dendrograms based on S_{AB} values were generated by UPGMA, implemented in the GelCompar software. An S_{AB} value of 1.00 indicates that the banding patterns for strain A are identical with that of strain B; S_{AB} values of 0.80–0.99 represent highly similar, but non identical, strains, and may suggest

the occurrence of microevolution in a single strain; and S_{AB} values below 0.80 represent unrelated strains[29,34-35]. *S. cerevisiae* chromosomal DNA standards was used as a reference. RAPD profiles were analyzed by GelCompar software Version 2.0 (Applied Maths).

Phenoloxidase activity

Melanin production was assessed visually by growing *Cryptococcus* sp on Niger seed agar (NSA) plates for up to 10 days and documenting the time necessary for pigmentation. Colonies were initially grey-brown but later turned black as melanization proceeded.

The extraction and quantification of phenoloxidase were made only in samples that possessed grey-brown and white colonies on Niger seed agar. *Cryptococcus* isolates were incubated previously in 2% malt extract. The suspension was centrifugated at 2500 rpm for 10 minutes. The cells were washed with Minimal Synthetic Medium without glucose and with ammonium sulfate and incubated in this medium for 5 h at 25^{o}C, with agitation. Glass beads (0.45 to 0.5mm) were added for the rupture of the cells and the whole was centrifuged at 4500rpm for 45 minutes. The cell wall fraction in the pellet was washed with PBS. The activity of the phenoloxidase enzyme was evaluated by the absorbance of the dopachromo and 5,6 dihydroxyindole at 480 and 300nm, respectively. These are produced by the oxidation reaction of the enzyme in the presence of 1mM L-norepinephrine[38].

Electrophoretic karyotyping

Karyotype analysis was done only in samples that was quantified the phenoloxidase, by contour-clamped homogeneous electrophoresis (CHEF). *C. neoformans*

chromosomal DNA plugs were prepared by mixing a protoplast suspension with 1.4% low-melting-point agarose solution (Bio-Rad) to yield a final agarose concentration of 0.7%. Electrophoresis was performed in a CHEF DRII variable-angle pulsed-field electrophoresis system (Bio-Rad) in 0.5 × TBE buffer (0.044M Tris-HCl, 0.044M boric acid, and 0.001M EDTA [pH8.0]) at 10^{o}C. Gels were run for 30 h with a switch time of 60–120s. *Saccharomyces cerevisiae* chromosome DNA molecular weight markers (Bio-Rad) were included in each gel as standards. After electrophoresis, gels were stained with ethidium bromide (final concentration 0.5 mg mL $^{-1}$) and photographed under UV light. Banding patterns were evaluated by visual inspection and analyzed using the ImageMaster VDS system (Amersham Pharmacia Biotech, Piscataway, NJ). The computer program GelComparII, version 2.0 (Applied Maths, Bel- gium), was used to determine the genetic relationship of the isolates. Similarity coefficients were calculated using the Dice algorithm, and cluster analysis was performed by the unweighted pair group method with arithmetic mean (UP- GMA)[29].

RESULTS

From a total of 40 cryptococcal isolates, 39 were recovered from 35 patients and identified as *Cryptococcus neoformans,* only one was *C. gattii*. The 5 patients with sequential isolates (each of them had 2 isolates) were identified *C. neoformans,* on both isolates. Thirty six (90%) belong to serotype A, three to serotype AD (7,5%) and one to serotype B (2.5%). Among the serial isolates, three patients (1, 7 and 30) presented same serotype (A) and two patients (6 and 34) had different serotypes. The first isolate of the patient 6 belong to serotype A and the second to the serotype AD, in addition to, in the patient 34, the first isolate was serotype AD and the second serotype A (Table 1).

The isolates 1742 (patient 3), 1785 and 1829 (both of patient 6) exhibited variable patterns of production of the phenoloxidase enzyme on Niger agar, with some colonies producing melanin in 48 hours and others later (7 to 10 days), these being denominated 1742r, 1785r and 1829r. The phenoloxidase enzyme and karyotype were assayed only in these six samples (Figure 1).

The data obtained by analysis of the amplification products with primer 6 were used to generate the dendrogram of the 40 samples, including the three samples with alteration in the production of the enzyme phenoloxidase. The similarity between different profiles varied from 0.53 to 1.0. The coefficient of similarity 1.0 represented identical strains. The initial threshold to discriminate the groups in this dendrogram was 0.80. The 27 representative profiles are located around this threshold. Considering the maximum index of similarity (1.0), the NTSys system of similarity coefficient analysis "Simple Match", found 5 possible clones, where one of these contained 4 identical isolates and the others 2 identical isolates each. Eight other groups, with coefficient of similarity 0.97, contained samples highly correlated genetically. The 40 isolates of *C. neoformans* were distributed in 6 clusters (I to VI) and these contained in 13 subgroups (Ia, Ib, IIa, IIb, IIIa, IIIb, IVA, IVb, IVc, Va, Vb, Via, Vib), and these were further subdivided into subtypes a', a'', b' e b'', most of them being placed between the coefficients of similarity 0.90 and 0.97. The isolate 143/96, belonging to the variety *gattii*, was totally discriminated, with coefficient < 0.7. All the serial isolates belonging to the same serotype A were highly correlated or considered to belong to the same clone. The serial isolates that belonged to different serotypes, showed marked differences in the subtyping (Figure 2).

Among patients who had serial samples of the same serotype, patient 1 (1269/1342) and 30 (167/42) presented the same genotypic profile, being O and K,

respectively, with S_{AB} value of 1.00. Patient 7 (1830/1850) showed S_{AB} value 0,9-0,97, with highly phylogenetic similarity. The results of the dendrogram demonstrate that the both serial samples belonged to the same clone (Figure 2).

The genetic profile of the serial samples with different serotypes was different. The patient 6 (1785/ 1829) sample showed the H profile, serotype A and AB profile for serotype AD; Ia' and Va, respectivel, with S_{AB} value < 0,7. A similar event occurred with patient 34 (34/104), with serotypes AD and A, and genotypic profile P and U, respectively and S_{AB} value 0,7-0,8 (Figure 2).

The analysis of the samples with different production patterns of the enzyme phenoloxidase showed variation in the genotypic profiles (1742 - genotypic profile F and 1742r - genotypic profile V; 1785 - genotypic profile H and 1785r - genotypic profile E; 1829 - genotypic profile AB and 1829r - genotypic profile W) (Figue 2). The electrophoretic karyotyping corroborates these data. The isolates 1742 and 1742r (patient 3), 1785, 1785_r, 1829 and 1829r (patient 6) were heterogeneous with respect to karyotyping, 6 different profiles being found. The number of chromosomes varied of 10 to 11 bands and their sizes varied between 0.7 and 2.8 Mb. The six profiles were classified A to F and the analysis of the isolates 1829 and 1829r (with early and late production of phenoloxidase) demonstrated the widely genetic variation (Figure 3).

The samples were not correlated with their respective samples with alteration in the production of the enzyme phenoloxidase. The lowest genetic distance was found between samples 1742 and 1742r, belonging to subtype IIIa and IIa, respectively. Sample

1785 was subtyped Ia' and 1785r as IIIb. In subtype Va the sample was found 1829 and in subtype I the sample 1829r (Figure 2).

Of the 40 samples, only 38 were evaluated for sensitivity to antifungal drugs. Both samples sequences from patient 6 did not growth in RPMI 1640 medium supplemented with 2% glucose nor in YNB, with and without 2% dextrose.

The MIC values obtained with the 38 isolates varied from 4 to 32 μg mL^{-1} for fluconazole, from 0,125 a 0,5 μg mL^{-1} for itraconazole, from 4 to 64 μg mL^{-1} for 5-FC and from 0,25 to 1,0 μg mL^{-1} for amphotericin B, these values being calculated at 50% inhibition for fluconazole, itraconazole and 5-FC and 100% inhibition for amphotericin-B.

Regarding the susceptibility profile of the *C. neoformans* isolates to fluconazole, the MIC_{50} showed that 8,5% and 46% presented 8 and 16 μg mL^{-1}, respectively. To itraconazole, 64,1% presented 0.25 μg mL^{-1} for MIC_{50}. Already, the values for 5-FC were between 4 and 8 μg mL^{-1}, with 33,3% and 48,7%, respectively. It was observed the 74,4%

of the isolates presented MIC_{100} of 0.5 μg mL^{-1} and 23,1% of 1 μg mL^{-1} for amphotericin-B.

In this study, most of the isolates were considered susceptible with dose-dependence to the drugs fluconazole and itraconazole, with MICs varying in the range 16-32 μg/ml and 0.25-0.5 μg/ml, respectively.

Patients with serial samples (1, 3, 7, 30 and 34), with the same and different serotypes, did not present significant changes in the MICs of the four drugs tested. Patients 1, 7 and 30 presented serotypes A for both samples. The second sample from patient 1 showed increased in one dilution for 5FC and amphotericin B. Already, the first sample of patient 7 presented greater MIC for fluconazole and 5FC in the first sample when compared to the second sample. For the drugs itraconazole and amphotericin B, the second sample showed a higher MIC (one dilution). Patient 30 presented the same MICs of the drugs fluconazole, itraconazole and 5FC in both samples. However, the MIC for amphotericin B was higher in the first sample (a dilution when compared to the second sample). The second samples from patient 34 (serotype A) showed increased dilution in MIC for the drugs fluconazole, itraconazole and 5FC when compared to the first sample (serotype AD). MIC for amphotericin B was the same for both serotypes.

As for the samples with alteration in the production of the enzyme phenoloxidase, only those obtained from patient 3 were evaluated for the susceptibility profile. Patient 3 presented a sample with late production of the enzyme phenoloxidase (1742 and 1742r). The MICs for the drugs tested were identical.

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The only isolate of the species *gattii* exhibited MIC_{50} of 16 μg mL for

-1 -1

fluconazole; 0.25 μg mL for itraconazole; 4 μg mL for 5-FC and MIC_{100} of 1.0 μg

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mL for amphotericin-B. This species showed dose-dependence for fluconazole and

itraconazole.

The MIC50 and MIC90 for fluconazole, 5-FC, itraconazole and amphotericin-B of

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the 38 analyzed isolates, gave the same values, being 16 μg mL , 8 μg mL , 0,5 μg

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mL and 0,5 μg mL , respectively.

DISCUSSION

The incidence of cryptococcal meningitis has increased after the pandemic of AIDS and despite availability of HAART continues to be the most common cause of opportunistic meningitis in many countries. All samples analyzed in this study were from HIV patients[13,37-40]. In Brazil, mortality by cryptococcosis was the thirteenth cause of death, with high mortality rate compared to other countries in Latin America [9,12].

In this study, the majority of samples were from serotype A and just one patient was infected by the species *gattii*. *C. neoformans* species complex (*var. grubii*, serotype A) has worldwide distribution, associated with pigeon and other bird droppings and soils

contaminated, and is prevalent in different regions of Brazil [5,39,41-44]. Cryptococcosis caused by *C. gattii* is endemic in northern and northeastern Brazil [10,39,45,46].

The resistance to antifungal drugs develops due to mutations that occur naturally in yeast cells, with drugs acting as selective agents by naturally selecting for cells without mutations. Prolonged use of antifungal drugs leads to the development of resistance which is evaluated by *in vitro* susceptibility tests. The study of the genetic mechanisms of resistance has been a tool for developing new diagnostic and therapeutic strategies[16,41,42,47].

The Minimal inhibitory concentration (MIC) in this study was determined by microdilution method M27-A3, reference methods by Clinical Laboratory Standard Institute (CLSI) for testin the susceptibility of *C. neoformans*. Studies had failed to find correlation between MICs from CLSI-methods and treatment outcome for AMB and FLC. Others techniques have been developed like E-test strips, disk diffusion, and time kill curves (TKC)[9]. Studies have shown divergence in results when compared with E-test, these may have more clinical relevance[45,47,48].

The highest MIC (32 μg/ml) for FLZ was observed in three samples, similar to the results presented by Rocha et al.[40] in isolates from clinical sources in Amazonas, Northern-Brazil. Tsujisaki et al.[43] evaluated the sensitivity profile of *Cryptococcus* isolated from clinical samples in Central-West Brazil against fluconazole, itraconazole, voriconazole and amphotericin B. All isolated samples were sensitive to the antifungal agents tested.

Aguiar et al.[37] evaluated strains of *Cryptococcus* isolated from patients in Southeast region of Brazil. All the isolates showed susceptibility to the tested antifungals

(fluconazole, voriconazole, amphotericin B and 5-flucytosine). A similar result was reported by Favalessa et al[49] in Midwest Brazil.

Worldwide studies have reported *Cryptococcus neofomans* isolated from clinical samples with increase in MIC for fluconazole[50]. Arechavala et al.[8] showed samples of *Cryptococcus neoformans* isolated from patients with cryptococcosis in Buenos Aires, Argentina with criptococosis. The study demonstrated samples with resistance profile to amphotericin B and fluconazole. However, Trpkovi et al.[51] analyzed clinical samples isolated from patients with HIV and non-HIV in Serbia, and unlike the results presented in this study, 29% of the samples were resistant to fluconazole, 12.9% to 5FC and 3.2% to itraconazole. No amphotericin B resistant sample was found.

C. gattii presented low MICs with all drugs tested. The results of antifungal susceptibility testing of *gattii* species, are variable, depending on geographic origin. Brazil is a large country and MICs reported from midwestern, southern and northeastern regions are different[42,47,49].

The HIV patients have a high probability of recurrence of cryptococcal meningoencephalitis even with successfully treated, which can be evidenced by the absence of clinical symptoms and sterilization of cerebral spinal fluid (CSF). The relapse due to persisting infection, is a phenomenon that the antimicrobial drugs impose strong selection pressure on pathogens, contribute to microevolution, leading to resistance [52,53]. The relapse phenomenon was confirmed in patient 1, 30 and 7. Both presented the same serotype and genotypic profile in the serial samples. The infection with a new isolate was confirmed in patients 6 and 34, both by serotyping and by analysis of phylogenetic similarity presented in the dendrogram. However, there was no change in susceptibility to the drugs tested.

Study developed by Rhodes et al[28] corroborated with those results. However, both in relapse infections of the original isolates or with a new isolate, the fourfold increase in FLC MIC was observed. It is essential the tests that evaluate the susceptibility of Cryptococcus, isolated in cases of relapse or recurrence, for the correct management of the treatment, allowing to compare the minimum inhibitory concentration with the original isolate, evaluating the decrease of the susceptibility[2].

Melanin is a powerful antioxidant, found in the fungal cell wall, with capacity to absorb free radical and to decrease the pore size that contribute to acquired resistance against to the amphotericin B and caspofungin[19,20,2,54]. It is undoubtedly that melanin interferes in susceptibility to antifungal drugs, but more researchs are need to elucidate the anti fungal mechanism of melanin[18].

Any conclusion about the influence of melanin on the susceptibility profile of the drugs tested in this study is early. From samples 1742, 1785 and 1829 with their respective samples with the production of the late phenoloxidase enzyme, 1742r, 1785r and 1829r respectively, only samples 1742 and 1742r could be evaluated in the drug susceptibility profile, with the same.

Grossman and Casadevall[47] suggest that the lack of correlation between FLC and AMB MICs with treatment outcome could be explained by the physiological conditions of the yeast in the host and *in vitro* tests. The main conditions are: absence of melanization in the RPMI broth, size of the polysaccharide capsule which is higher *in vivo* when *in vitro* conditions, presence of *titan cells* in the host but not in cultures, and others expressions changes[55,56].

By dendrogram analysis, it can be seen that the samples of *C. neoformans* from different regions of the state of Sao Paulo presented a high genetic correlation (clusters I

to IV), S_{AB} values of 0.80–0.99. Pedroso et al (2012) showed a good discriminatory between different species of *Cryptococcus* using RAPD.

Andrade-Silva et al[57] evaluated environment and clinical isolates of *C. neoformans* by RAPD and correlate the genetic profiles with virulence factors and antifungal susceptibility patterns. Most clinical isolates cluestere homogeneously similarly, and no correlation between virulence factors or antifungal susceptibility profile with the obtained RAPD profiles was observed. Corroborating with the data found in the present study.

Rocha et al.[40] reveal a clonal population for *C. neoformans* isolates from clinical sources in Amazonas, Northern-Brazil, using MLST. Favalessa et al.[49] (2014) using URA5-RFLP showed a predominant genotype affecting HIV-negative individuals in Cuiabá.

Different molecular techniques have demonstrated a high genetic similarity between clinical samples analyzed in worldwide, including Brazil. The result of this study is in agreement with Brazilian and worldwide studies, using both RAPD and other molecular techniques. In the same way, the drugs profile susceptibility tested did not constrain with data referring to the analyzes performed in Brazil. As reported in other studies, there is correlation between genotype correlation and virulence factors expression, a fact that was not proven in this study, due to the restricted number of samples to be analyzed.

Studies to evaluate the relationship between molecular profile and phenotypic characteristic, such as virulence factors, resistance of antifungal drugs are necessary to search for new molecular markers for genotyping, pathogenicity and drug susceptibility.

The current techniques that assess the susceptibility profile to antifungal are based on phenotypic characteristics that may lead to mistakes by the non-gene expression in the envirommment that develops the technique. More accurate and early diagnosis, efficient clinical support, and proper antifungal therapy are essential to reduce death and sequels caused by cryptococcosis.

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Figure 1 – Representative graph of the phenoloxidase quantification from the samples of patients 3 and 6 that presented different standards in Niger agar.

Table 1 - Characterization of the 40 samples from 35 patients, serotype, phylogenetic profile and karyotype.

Figure 2 - Dendrogram of the 43 samples of *C. neoformans* obtained by RAPD analysis.

Figure 3 - Representative photo of the karyotypic profiles of *C. neoformans* samples with different patterns in the production of the enzyme phenoloxidase.

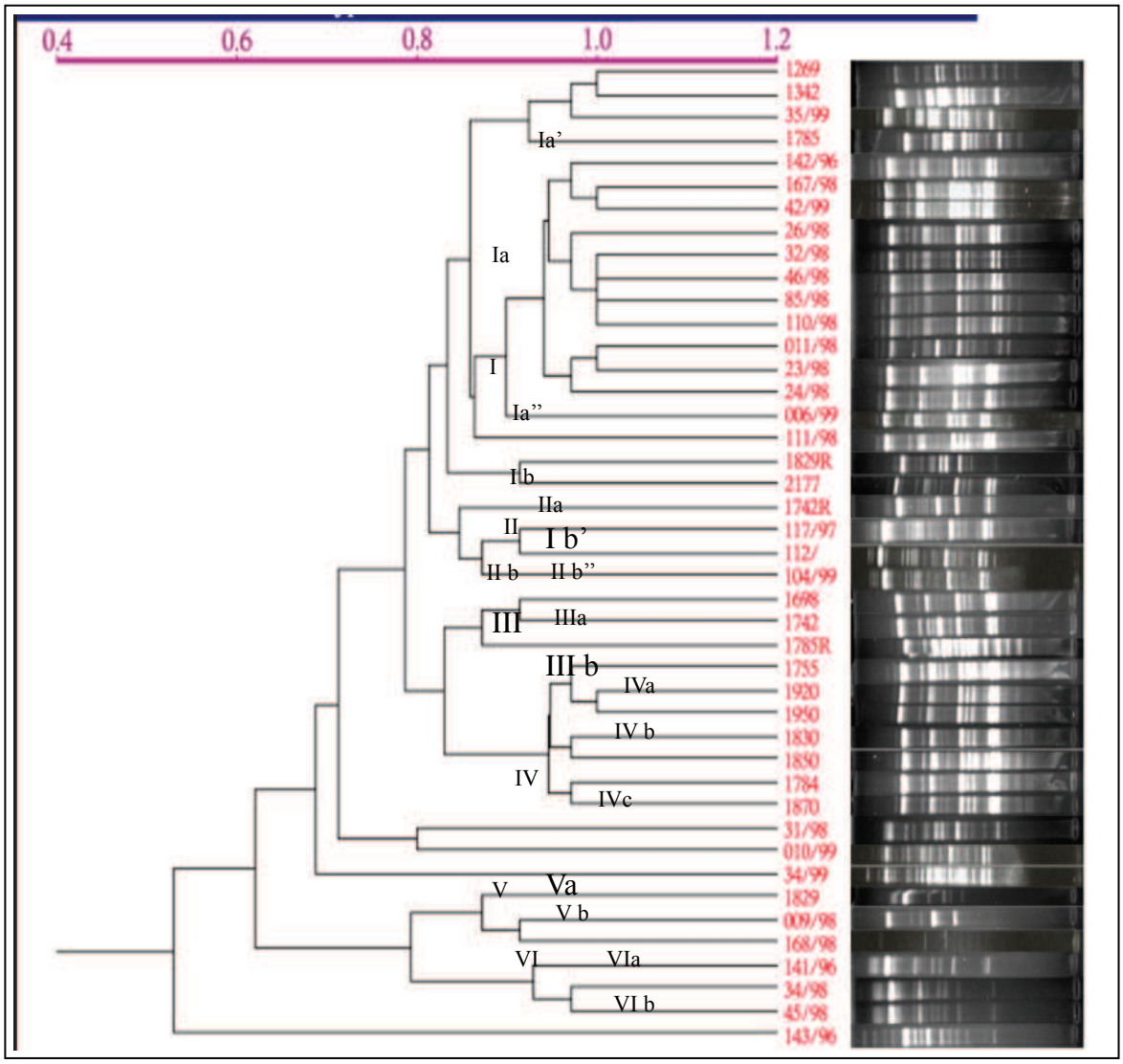
1-1742 sample; 2-1742r sample; 3-1785 sample; 4-1785r sample; 5- 1829 sample; 6-1829r sample; 7- *C. neoformans* (ATCC 90012) sample.

Table 1

Patient	Sample	Serotype	RAPD profile	Karyotype
1	1269	A	O	-
	1342	A	O	-
2	1698	AD	G	-
3	1742	A	F	EKA
	1742_R	A	V	EKB
4	1755	A	A	-
5	1784	A	D	-
6	1785	A	H	EKC
	1785_R	A	E	EKD
	1829	AD	AB	EKE
	1829_R	AD	W	EKF
7	1830	A	C	-
	1850	A	B	-
8	1870	A	C’	-
9	1920	A	B'	-
10	1950	A	B’	-
11	2177	A	X	-
12	141	A	AA	-
13	142	A	I’"	-
14	143	B	Y	-
15	117	A	J	-
16	9	A	AC	-
17	11	A	I’"	-
18	23	A	I’"	-
19	24	A	L	-
20	26	A	I’	-
21	31	A	S	-
22	32	A	I	-
23	34	A	Z’	-
24	45	A	Z	-

25	46	A	I	-
26	85	A	I	-
27	110	A	I	-
28	111	A	R	-
29	112	A	Q	-
30	167	A	K	-
	42	A	K	-
31	168	A	AD	-
32	6	A	M	-
33	10	A	T	-
34	34	AD	P	-
	104	A	U	-
35	35	A	N	-

Figure 2


0.4
0.6
0.8
1.0
1.2
1269
1342
35/99
1785
142/96
167/98
42/99
26/98
32/98
46/98
85/98
110/98
011/98
23/98
24/98
006/99
111/98
1829R
2177
1742R
117/97
112/
104/99
1698
1742
1785R
1755
1920
1950
1830
1850
1784
1870
31/98
010/99
34/99
1829
009/98
168/98
141/96
34/98
45/98
143/96
Ia'
Ia
I
Ia"
I b
IIa
II
I b'
II b
II b"
III
IIIa
III b
IVa
IV b
IV
IVc
V
Va
V b
VI
VIa
VI b

ARTIGO CIENTÍFICO 2

Brazilian Archives of Biology and Technology

Human pathogenic yeasts isolate in environment with azoles resistance

Running title: *Candida* spp resistance profile in environment

ABSTRACT

Candida species is the most important human fungal pathogen occupying natural ecosystems. The aim of the present study was investigated the Candida species in wood and evaluated the antifuganl susceptibility profile. Four hundred samples were analyzed from 2015 to 2016. C. tropicalis, C. albicans, C krusei, C. gladrata, C. parapsilosis, C. guillermondii and C. laurentii were isolated from wood. High percentage of samples showed resistance profile to fluconazol and itraconazol. Clinical study demonstrated increased in Candida non-albicans with low susceptibility, corroborate with these results. More investigate is necessary to understand the impacting of environmental conditions on natural history of pathologies.

Key words: *Candida*, susceptibility, wood, environmental, azoles.

INTRODUCTION

Yeasts are unicellular fungi that have a crucial role for humanity, with industrial, agricultural, environmental, scientific applications and public health. These fungi occupy various natural ecosystems, such as aquatic environments, soil and organic matter. The diversity and density of yeasts in the phyllosphere are linked to geographical area, climatic conditions, season and plant species which affects the community[1]. *Candida albicans* is the most prevalent human fungal pathogen asymptomically inhabiting diverse host niches, able to cause disease in both, immune-competent and immune-compromised individuals, is considered commensal of animals, yet it is occasionally isolated from trees, shrubs, and grass. Biofilms are a major threat to human health as they are resistant to the host immune system and antibiotics. Thus, *Candida* biofilms on indwelling medical devices and mucosal tissues are one of the most common causes of systemic lethal infections[2,3].

Fungal endocartiditis episodes represented healthcare-associated infections, accounts for 2% of overall infective endocarditis, associated with high mortality rates (30-80%). The pathology were caused most frequently by *Candida* spp, and non-albicans predominated, such as *C. parapsilosis*, *C. tropicalis*, *C. psilosis*, *C. glabrata* and *C. lusitaniae*[4,5].

Candidemia is a serious bloodstream infection with mortality rates exceeding 40% in Brazilian Hospital. Epidemiologic studies shown that *C. albicans*, *C. tropical* and *C. parapsilosis* account for the majority of candidemia episodes, followed by *C. glabrata*[6-10].

Vulvovaginal candidiasis (VVC) and onychomycosis are common fungal infections in Brazil, with emergency by non-albicans as pathogen. VVC is the second most common cause of genital infection in women of reproductive age, represents a problem of global importance public health. Given the high incidence of onycomycosis in the country and the difficult to treat with appropriate medication both pathologies, epidemiological studies have shown different resistance rates of *Candida* spp[11,12].

The increase in the incidence and spread of emerging fungal infections in the world over the last decades is associated with individuals factors, such as reduction in the host's natural defenses, with fungal factors, such as antifungal resistance and with environmental conditions, such as natural disasters that changing the environmental conditions and alter the fungal populations. These factors highlight the importance of environmental factors on the dispersion of fungal diseases[13,14].

The Brazil presents diversity in the ecosystem and little attention has been brought to environmental isolates. There are few environmental investigations of yeasts of medical interest in Brazil, which are necessary to understand the natural history of the disease.

The aim of the present study was investigated the population dynamics of *Candida* species of medical interest throughout the environmental and and to establish (i) antifungal susceptibility profile and (ii) correlation with influence of climatic conditions

MATERIALS AND METHODS

Yeast isolation

Decayed wood samples (n= 400) of material decomposing in the hollows of the trees were collected from twenty five different areas in Votuporanga City, São Paulo State, Brazil (20o25’22"S 49o58’22’’W).

The environmental isolates was obtained from five different geographical points of an urban city: north, south, east, west and central. The criterion of choice was a wooded area with a large population movement, selecting 5 trees in each region, totaling 25 trees analyzed. Four samples of each tree were standardized in each collection, in four climatic seasons of the year (Figure 1).

The samples were collected with sterile tweezers and spatulas and stored in sterile plastic bags, transported in isothermal boxes with room temperature to laboratory. The samples was processed within 2h after harvesting.

Wood samples (5g) were placed in 250 mL flasks containing 50 mL of saline solution sterile with chloramphenicol 0,04% and shaken on a rotatory shaker for 2 h at 25oC. The washing were serially diluted in saline solution sterile up to 1/512 and 0.1mL of each dilution was spread on the Niger agar and Sabouraud Agar, incubated at 30ºC to 7 days and room temperature to 15 days, respectively.

The morphological and physiological characteristics of yeast cultures were examined by the methods described by Kurtzman et al.[15]. Strains were identified according to Kurtzman et al.[16]. Furthermore, yeast colonies were plated on Chromagar Candida (bioMerieux®) and the ID 32C systems (bioMerieux®) were used to assess growth on various carbon sources after incubation at 28ºC for 2 day.

Antifungal susceptibility test

The Kirby Bauer Disk diffusion method was performed according to CLSI standard M44-A2 to determine antifungal susceptibility of the *Candida* species isolates[17]. Discs impregnated with known concentrations of antifungal agents were obtained. The following antifungal agents were used: fluconazole (FCN; 25 mcg), ketoconazole (KCA: 50mcg), itraconazole (ITR: 10mcg), amphotericin (AMB: 100mcg). Incubation was done at 30ºC. Diameters (in mm) of the growth inhibition zones were measured after 24 hours.

RESULTS AND DISCUSSION

Antifungal resistant *C. albicans* and *non-albicans* were isolated from wood in the North West of São Paulo State during 2015 and 2016. Four hundred samples from six plants: *Bauhinia variegata, Caesalpinia pluviosa, Linicania tomentosa, Tabebuia heptaphylla, Delonix regia, Schinus molle* were investigated for the yeast species. Of the 400 samples analyzed, 88 (22%) were positive for yeasts and 76 showed *Candida* spp. Some decayed wood samples showed the isolation of more than one type of yeast, totalizing the identification of 91 isolates, being 77 of *Candida* spp. (Table 1).

Table 1 – Relation of frequency of yeast species in 91 isolates of 88 decayed wood samples.

Number of Isolates	Yeast species	Frequency of yeast species (N/%)
91	*Candida tropicalis*	25/27,5%
	Candida parapsilosis	6/6,6%
	Candida krusei	11/12,1%
	Candida albicans	18/19,8%
	Candida glabrata	10/11%
	Candida laurentii	3/3,3%
	Candida guillermondii	4/4,4%

	Trichosporon beigelli	6/6,6%
	Rhodotorula rubra	4/4,4%
	Rhodotorula glutinis	2/2,2%
	Sporobolomyces salmonicolor	1/1,1%
	Blastoshizomyces capitatus	1/1,1%

Linicania tomentosa presented a higher percentage of positive samples, 23 positive samples of *Candida* spp in 48 samples evaluated (47,9%). However, as there was no fixed pattern of samples per tree species, the frequency data of each tree species are detailed in table 2.

Table 2 – Correlation of tree species and positive samples of *Candida* spp.

Tree species	Frequency of tree species in 400 samples (N/%)	Frequency of tree species with positive samples (N/%)
Bauhinia variegata	128/32%	9/7,0%
Caesalpinia pluviosa	128/32%	30/23,4%
Linicania tomentosa	48/12%	23/47,9%
Tabebuia heptaphylla	64/16%	8/12,5%
Delonix regia	16/4%	3/18,8%
Schinus molle	16/4%	3/18,8%

Each region of the city was evaluated in the four seasons of the year. It was standardized to collect 20 samples at each station for each region. When correlating the season of the year and the isolation of *Candida* spp, it can be verified that the greatest number was realized in the summer (Table 3).

Table 3 – Correlation of season, region analysed and percentage of *Candida* spp isolates.

REGION	SEASONS	FREQUENCY OF POSITIVE SAMPLES (%)
WEST	SUMMER	15%
	WINTER	10%
	SPRING	-
	AUTUMN	50%
CENTRAL	SUMMER	95%

CENTRAL	WINTER	25%
	SPRING	20%
	AUTUMN	5%
NORTH	SUMMER	-
	WINTER	-
	SPRING	25%
	AUTUMN	30%
EAST	SUMMER	70%
	WINTER	5%
	SPRING	5%
	AUTUMN	20%
SOUTH	SUMMER	-
	WINTER	5%
	SPRING	-
	AUTUMN	-

Candida tropicalis is a pathogenic yeast with worldwide recognition as the second or third more frequently isolated species in Latin America, for both superficial and systemic infections. This yeast belongs to the normal human microbiota and may be acquired from either endogenous or exogenous sources. Besides belonging to the human normal microbiota, *C. tropicalis* may be found in other warm blood animals and in the environment, including water and sand of beaches[18,19].

Studies showed that *C. tropicalis* is well adapted to the environmental conditions and can remain viable in nature for longer than *C. albicans.* Zuza-ALves et al.[19] demonstrated that geographic clustering as a function of climatic seasonality among environmental isolates of *C. tropicalis* in the northeastern region of Brazil. The high genetic variability is not only to microevolution promoted by prolonged colonization of microorganisms well adapted to environment and the relative maintenance of the population structure within the same season.

No *Candida* spp resistance to amphotericin B was found. Among the *Candida* species evaluated, *C. guillermondii*, *C. krusei*, *C. albicans* , C. *glabrata* showed a high resistance profile. The susceptibility profile to the tested antifungal is described in table 4.

Yeast	**DRUG**											
	FLUCONAZOLE			**ITRACONAZOLE**			**KETOCONAZOLE**			**AMPHOTERICIN**		
	S	**SDD**	**R**	**S**	**SDD**	**R**	**S**	**SDD**	**R**	**S**	**SDD**	**R**
	N/F%			**N/F%**			**N/F%**			**N/F%**		
Candida tropicalis	**24/96**	**0/0**	**1/4**	**24/96**	**0/0**	**1/4**	**25/100**	**0/0**	**0/0**	**25/100**	**0/0**	**0/0**
Candida parapsilosis	**4/67**	**0/0**	**2/33**	**4/66**	**1/17**	**1/17**	**6/100**	**0/0**	**0/0**	**6/100**	**0/0**	**0/0**

Candida krusei	**0/0**	**0/0**	**11/100**	**8/73**	**2/18**	**1/9**	**10/91**	**0/0**	**1/9**	**11/100**	**0/0**	**0/0**
Candida albicans	**9/50**	**0/0**	**9/50**	**12/67**	**0/0**	**6/33**	**14/78**	**0/0**	**4/22**	**18/100**	**0/0**	**0/0**
Candida glabrata	**3/30**	**3/30**	**4/40**	**4/40**	**4/40**	**2/20**	**10/100**	**0/0**	**0/0**	**10/100**	**0/0**	**0/0**
Candida laurentii	**3/100**	**0/0**	**0/0**	**2/67**	**1/33**	**0/0**	**3/100**	**0/0**	**0/0**	**3/100**	**0/0**	**0/0**
Candida guillermondii	**0/0**	**0/0**	**3/100**	**1/33**	**0/0**	**2/67**	**2/67**	**1/33**	**0/0**	**3/100**	**0/0**	**0/0**

Table 4 – Profile of antifungal suscetibility of *Candida* spp isolates from decayed wood.

Candida non-*albicans* species were more commonly isolated from breakthrough candidemia cases, including species resistant to earlier used antifungal drugs, the majority being azoles. *C. glabrata*, *C. parapsilosis* and *C. tropicalis* resistant to triazoles and echinocandins[7,8].

Candida glabrata has been reported as a second cause of candidiasis in medical centers in United States, Australia and European countries. The emergency of this pathogen also has been showed in Brazil[20-25]. Genetic instability by several mutations play a role in the multidrug-resistant (MDR) *C. glabrata* emergence, showing low azole susceptibility, polyene and echinocandins resistance[26,27].

Wound and urinary infections, peritonitis and candidemia by *Candida guilliermondii* have been reported. The intrinsic decreased susceptibility to azoles and echinocandins is related to a high proportion of patients receive inappropriate antifungal treatment[28,29]. The phenomenon has been reported to others *Candida* spp, such as *Candida krusei*, *Candida lusitania* and *Candida kefir*[30].

Savastano et al[23] evaluated the incidence of the *Candida* species from the environment and health practitioners in a Brazilian Hospital. *C. glabrata* was the predominant species, followed by *C. parapsilosis* and *C. tropicalis.* All sample of *C. albicans* presented high susceptibility to the drugs tested. *C. krusei*, *C. glabrata*, *C. parapsilosis* and *C. tropicalis* presented a decreased susceptibility to azoles.

The surface properties of wood can significantly influence the quantity and fungal adhesion. The yeast adhesion is the first step of biofilm formation, which may be responsible for the increase of the genetic expression resulting in different phenotypes[31]. Tomicic et al.[32] demonstrated the *C. albicans* and *C. glabrata* adhered better to wooden surfaces than *C. parapsilosis* and *C. krusei*, corroborating the research datas (percentage difference of the isolated species).

The broth micro dilution methods (CLSI and EUCAST) is the reference to determined the antifungal susceptibility profile of *Candida* spp isolates. Being this laborious and costly technique several commercial assays (Vitek2, Etest, Sensititre Yeastone) have been used in clinical laboratories to provide the antifungal susceptibility profile of *Candida* spp isolates. Although discrepant results have been described for commercial assays when compared with the reference methods. Thus, this search opted for the disk diffusion CLSI standards to evaluate the *Candida* resistance in environment[33,34,35].

C. albicans is only rarely isolated from plants or other environmental substrates, on gorse flowers and myrtle leaves in Portugal, on grass in New Zealand, on tulip tree in the Cook Islands and on oak trees in an ancient wood in the United Kingdom[2,36]. Others *Candida* species such as *C. tropical* and *C. parapsilosis* live on trees[37,38].

Bensasson et al.[2] compared the genome sequence of *C. albicans* isolated from oak trees to the clinical strains and showed they are similar. The oak strain was more closely related to strains from humans and other animals that to strains from other oaks, suggesting that the high genetic diversity of *C. albicans* from old oaks can live in the environment for long period of time.

Studies in Brazilian aquatic environments showed strains of *C. albicans* resistance to fluconazole, itraconazole and amphotericin B[39-41]. The resistance profiles against itraconazole and fluconazole of *C. tropicalis*, *C. parapsilosis*, *C. glabrata* in water ecosystems was also observed[39,42].

The hypotheses about increasing the resistance of strains of *Candida* isolated from the environment is related to the indiscriminate use of antifungals and fungicides of azole class in agriculture and population, that results in the contamination and acting as selecting agents in the environment, since remaining and accumulating, allowing a gradual selection of resistance mechanisms by the microorganisms[41,43,44].

Another hypothesis has been related to anthropogenic activity and the degraded environment, promote altered expression of genes which occasionally occur in pathways related to resistance, such as efflux pumps[42].

Epidemiological studies in Brazil has been described the increasing of azoles resistance among clinical isolates of the *C parapsilosis* and *C. tropicalis*[45,46]. The aim of this study was to determine antifungal susceptibility of *Candida* species isolated from trees in cosmopolitan areas. The results demonstrated a high percentage of resistant and dose dependent *C. parapsilosis* to the azoles (fluconazole and itraconazole). However, *C. tropicalis* samples showed sensibility profile to the antifungals tested.

Monapathi et al[47] demonstrated that all the environmental isolates *Candida albicans* were resistant to azoles, being that efflux pump genes were detected in 60% of the isolates. Phylogenetic analysis showed high similarity between from environmental and clinical isolates. These findings are alarming and could result in cross-resistance to various drugs.

The resistance to amphotericin B is shown to be rare, however have been demonstrated by Milanezi et al.[41] in environment samples. Polluted environment can induce an oxidative response in microorganism, that result of adaptation to this stress. The overexpression of superoxide dismutase and catalase was observed in *C. albicans* and *C. dublinienses* strains resistant to amphotericin B and fluconazole[48,49].

Scientific reports have demonstrated the presence of numerous *Candida* species isolated in Brazilian and in native forests the world. These are unusual in causing human pathologies but show close phylogenetic relation with *C. parapsilosis*[50-53].

Most fungi in environment grow ate 12-30^{o}C suffering decline in growth and viability at temperatures above 30^{o}C. The high mammalian body temperatures are sufficient to minimize the replication of fungal. The global warming is a environmental pressures that changes the pathogen ecology, and contributes in stronger pathogens with advanced virulence mechanisms to adapted in harsher environmental conditions, by selecting thermotolerant fungal and facilitate the interaction with human populations, leading to the emergence of novel fungal pathogens[54,55].

The microevolution provide an ability to adapt rapidly to fungi during the colonization of human hosts, such as escaping to recognition and destruction by the host immune system. In addition, could contribute to emergence of resistance to antifungal agents[56,57].

Currently, the *Candida auris* has emerged as a multidrug-resistant *Candida* species associated with high rates of mortality in world since 2008, date of the first description as pathogen human. Corroborate with rapid spread as new public health problem[58-60].

Bastos et al.[61] showed that *Cryptococcus* spp tebuconazole exposure caused *in vitro* resistance to clinical azoles (fluconazole, itraconzaole and ravuconazole). These results support the idea tha agrochemical can affect human pathogens present in environment.

Rocha et al[62] demonstrated the influence on resistance to fluconazole, itraconazole and voriconazole in *Candida parapsilosis* exposured to the tetraconazole and the organophosphate malathion, both widely used in farming activities. Studies have suggested that the azole resistance in veterinary and environmental strains can be acquired in the environment[62-64].

Currently, *Candida utilis* and *Candida lipolytica* that were considered enviromental pollutants are identified as agents of fungemia, onychomicosis and systemic disease. The same with *Candida lusitaniae*, *Candida holmii*, *Candida norvegensis* and *Candida valida*[65].

Understanding how impacting environmental factors have on the emergence of pathogens is necessary. Several doubts need to be elucidated, (i) will global warming contribute to the emergence of new pathogens by natural selection? (ii) the indiscriminate use of antifungals in agriculture and livestock farming plays a crucial role in the resistant strains in the environment? (iii) what is the impact of the isolated resistant strains on the environment and the emergence of new diseases and / or new pathogens?

CONCLUSIONS

In summary, the *Candida* spp isolated in decayed wood samples of material decomposing in the hollows of the trees showed high correlation with the pathogenic yeasts

reported in epidemiological studies. The environment isolates of *Candida* spp were low susceptibility rate to azoles. Understanding the interaction of the environment with emerging pathogens is necessary for the prevention and promotion of collective health.

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Figure 1 - Scheme representative of the five geopraphical points of Votuporanga city were collected de environmental samples in four climatic seasons.

ARTIGO CIENTÍFICO 3

Arquivo Brasileiro de Medicina Veterinária e Zootecnia

Presença de *Candida albicans* e não *albicans* resistente aos antifúngicos azóis em frangos de corte

Presence of *Candida albicans* and non-*albicans* resistant to azole antifungals in broiler chickens

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RESUMO

A *Candida* spp é uma importante levedura patogênica para animais e humanos. Objetivou-se com o presente estudo avaliar o ecossistema das fezes de frangos de corte quanto a presença de leveduras de interesse médico, bem como o perfil de sensibilidade aos antifúngicos. Cento e oitenta amostras de fezes coletadas da cama de frango de corte de cinco aviários no período de 2015 e 2016, na região do Noroeste Paulista foram analisadas. As fezes foram suspensas em solução salina estéril com cloranfenicol e diluídas, com posterior plaqueamento em ágar Sabouraud e Niger. As leveduras foram identificadas pelas características bioquímicas. Das amostras analisadas, 23,33% apresentaram presença de *Candida* spp. As espécies isoladas foram: *Candida tropicalis*, *Candida albicans, Candia parapsilosis*, *Candida krusei*, *Candida zeylanoides*, *Candida rugosa* e *Candida pelicullosa*. Os isolados apresentaram elevado perfil de sensibilidade aos antifúngicos testados (fluconazol, cetoconazol, itraconazol e anfotericina-B). Somente 2/26 amostras de *C. tropicalis* e 1/6 de *C. albicans* foram resistentes a fluconazol e itraconazol, concomitantemente. Os galináceos têm importante papel da disseminação de leveduras de interesse médico aos humanos, compreender o processo adaptativo dos fungos patogênicos é fundamental para a história natural da doença, desenvolvendo ações preventivas que contribuam para promoção da saúde coletiva.

Palavras-chave: aves, *Candida* spp, antifúngicos, suscetibilidade, ecoepidemiologia

ABSTRACT

Candida spp is an important pathogenic yeast for animals and humans. The objective of this study was to evaluate the broiler feces ecosystem in relation to the presence of yeasts of medical interest, as well as the antifungal sensitivity profile. One hundred and eighty stool samples collected from the broiler litter bed of five poultry in the period of 2015 and 2016 in the region of Northwest Paulista were analyzed. The feces were suspended in sterile saline solution with chloramphenicol and diluted, after plating on Sabouraud and Niger agar. Yeasts were identified by biochemical characteristics. Of the samples analyzed, 23.33% presented *Candida* spp. The isolated species were: *Candida tropicalis*, *Candida albicans*, *Candia parapsilosis*, *Candida krusei*, *Candida zeylanoides*, *Candida rugosa* and *Candida pelicullosa*. The isolates had a high sensitivity profile to the antifungal agents tested (fluconazole, ketoconazole, itraconazole and amphotericin-B). Only 2/26 samples of *C. tropicalis* and 1/6 *C. albicans* were resistant to fluconazole and itraconazole, concomitantly. Understanding the adaptive process of pathogenic fungi is fundamental to the natural history of the disease, developing preventive actions that contribute to the promotion of collective health.

Key-words: birds, *Candida* spp, antifungals, susceptibility, ecoepidemiology.

INTRODUÇÃO

As aves domésticas e selvagens têm um importante papel na saúde pública como portadoras de patógenos tais como protozoários, fungos, bactérias e vírus. Vários estudos têm esclarecido a importância das fezes de aves como substrato para o crescimento de leveduras e fungos filamentosos, por apresentarem elevada concentração de compostos nitrogenado. Além disso, a presença de leveduras nas fezes das aves contribui para a dispersão ambiental (Mendes et al., 2014; Elhariri et al., 2015).

Nas últimas décadas, o mundo tem experimentado o aumento da incidência de doenças fúngicas. Dentre as leveduras, a *Candida* spp ocasiona patologias cutâneas e sistêmicas em animais e humanos. A emergência de novas espécies de leveduras como importantes patógenos humanos tem sido relatada. Atualmente, *Candida utilis* e *Candida*

lipolytica que antes eram consideradas poluentes ambientais são patógenos na comunidade. Assim como, *Candida lusitaniae*, *Candida holmii*, *Candida norvegensis* e *Candida valida* (Brilhante *et al.*, 2013; Araujo *et al.*, 2017; Reagan *et al.*, 2019).

A emergência de novas patologias está associada às alterações no hospedeiro e/ou no ecossistema do patógeno, tais alterações ambientais que implicarão na interação entre esses. A maioria dos fungos capazes de ocasionar infecções humanas são saprófitos ambientais que coabitam com outros microrganismos, e a patogenicidade deriva da capacidade de desenvolver estratégias de sobrevivência no ambiente sob estresse e em hospedeiros animais (Brown *et al.*, 2014).

As infecções desenvolvidas nas aves podem ser pela contaminação ambiental dos seus habitats. A fácil dispersão dos esporos fúngicos pelo ar, pode contaminar humanos, ocasionando micoses como onicomicose, otite, ceratomicose e fungemias (Elhariri et al., 2015; Mendes et al., 2014).

Dentro desse contexto, os animais domésticos e selvagens têm importante relação com o processo adaptativo evolucionário de fungos patogênicos (Losnak *et al.*, 2018). Entretanto, raros estudos têm se atentado ao problema ambiental como possível fonte de contaminação em humanos. O presente estudo objetivou avaliar a presença de leveduras de interesse médico em fezes de frangos de corte e correlacionar com o perfil de suscetibilidade aos antifúngicos.

MATERIAL E MÉTODOS

Cento e oitenta amostras de fezes (1-5g) coletadas da cama de frango de corte de cinco aviários no período de 2015 e 2016, na região do Noroeste Paulista, foram avaliadas. Os aviários mediam 17m de largura, 165m de comprimento e 2,65m de altura. Todos os aviários apresentavam em uma extremidade um exaustor e na oposta o silo para armazenamento da ração. Foi padronizado para a coleta, a divisão de cada aviário em 3 setores (cada um medindo 55 metros x 17 metros, totalizando 935m^2) e designados: setor do exaustor, setor do meio, setor do silo. Cada setor foi subdividido em 6 subsetores contendo aproximadamente 155m^2.

Os aviários foram avaliados em 2 situações: 1ª coleta, 7 dias após o recebimento dos pintos; 2ª coleta: entre 30 a 45 dias após a data da 1ª coleta. As amostras de fezes foram acondicionadas em sacos coletores estéreis e encaminhadas em caixas isotérmicas a temperatura de 22-25ºC ao Laboratório Didático de Análises Clínicas do Centro Universitário de Votuporanga – UNIFEV.

Após homogeneização dos sacos coletores, 5g de fezes foi homogeneizado em gral com pistilo esterilizado e acrescentados à solução salina estéril contendo cloranfenicol (0,4g/L), com agitação por 5 minutos. Posteriormente, após repouso de 10 minutos, 100µL foi transferido para um tubo estéril (P) e 100µL foi transferido para um tubo contendo 100µL de salina estéril (1/2). Após agitação, foram realizadas 2 diluições seriadas (1/4 e 1/8). 100µL de cada diluição foram plaqueadas no ágar Sabouraud Dextrose (Oxoid®) e ágar Niger (*Guizotia abyssinica*) incubadas à 30ºC por 24-48 horas e 10 dias, respectivamente. Colônias de leveduras foram avaliadas macroscopicamente e microscopicamente, pela coloração de Gram e tinta da China com posterior subcultivo em Chromoagar *Candida* (Himedia®). As leveduras foram identificadas bioquimicamente: hidrólise da uréia, assimilação de carboidratos e nitrogênio, resistência à canavanina e metabolização da glicina (ágar CGB) e ID32C (Biomerieux®) (Filiu et al., 2002 com modificações).

Após a identificação, os isolados foram avaliados quanto ao perfil de sensibilidade aos antifúngicos pela técnica de difusão em disco (CLSI, 2009). Quatro drogas foram testadas, anfotericina-B (CECON®), cetoconazol (CECON®), fluconazol (CECON®) e itraconazol (CECON®). *Candida albicans* ATCC 90028 foi utilizada como controle. Os diâmetros dos halos de inibição foram avaliados e de acordo com as instruções do fabricante considerados: anfotericina-B: sensível (S): >10mm, resistente (R): ≤ 10mm; cetoconazol: sensível (S): > 20 mm, dose-dependente (DD): 10-20mm, resistente (R): ≤ 10mm; fluconazol: sensível (S): >19 mm, dose-dependente (DD): 14-19 mm, resistente (R): ≤ 14 mm; itraconzaol: sensível ≥ 20 mm, dose-dependente (DD): 12-19 mm e resistente (R): ≤ 11 mm.

RESULTADOS E DISCUSSÃO

Das 180 amostras de fezes avaliadas, 23,33% (42) apresentaram crescimento de *Candida* spp com 46 isolados de *Candida* spp. A espécie *C. tropicalis* apresentou maior isolamento. As espécies isoladas com as respectivas frequências estão listadas na Tabela 1.

Tabela 1 – Descrição da frequência das espécies de *Candida* isoladas nas 180 amostras de fezes de frangos de corte analisadas.

Levedura	**180 amostradas analisadas**	
	42 amostras positivas (23,33%)	
	Número absoluto	**Frequência (%)**
Candida tropicalis	26	32,50%
Candida krusei	4	5%
Candida zeylanoides	2	2,5%
Candida rugosa	2	2,5%
Candida pelicullosa	1	1,25%
Candida albicans	6	7,5%
Candida parapsilosis	3	3,75%

Em humanos, a *Candida* spp é comumente encontrada na microbiota do trato digestório de indivíduos saudáveis e são responsáveis por mais de 80% das infecções nosocomiais (Colombo et al., 2013). A *Candida albicans* é um importante patógeno humano, comensal em humanos e na maioria dos animais de sangue quente (Bensasson *et al*, 2019); entretanto, nos últimos anos, dados epidemiológicos têm demonstrado um declínio dessa seguido do aumento das espécies não-*albicans*, mais especificamente a *Candida parapsilosis* e *Candida tropicalis* (Guinea, 2014; Vallabhaneni et al., 2016).

Comensalismo semelhante é encontrado nos animais, sendo que a taxa de colonização é dependente da espécie e criação. Entretanto, alterações na imunidade e barreiras anatômicas podem transformá-la em patógeno, com habilidade em infectar diversas espécies (Foster et al., 2013; Brilhante et al., 2016).

Alguns animais têm importante relação com as infecções fúngicas e pesquisar por patógenos humanos em amostras de animais é uma oportunidade para a eco-epidemiologia (Lonask *et al*., 2018). Assim, o conhecimento da microbita fecal das aves é essencial para identificar organismos que atuam como reservatórios para a transmissão de zoonoses

(Simi *et al*., 2018). As leveduras presentes como comensal nas fezes das aves podem ocasionar infecções em pacientes imunodebilitados na sociedade, e nesse contexto destaca-se a fungemia por a *Candida* spp. Estudos corroboram na hipótese que os galináceos têm importante papel na disseminação de leveduras para outros animais, humanos e meio ambiente (Cafarchia *et al*., 2018).

A *Candida albicans* é um patógeno oportunista e ocasiona uma variedade de doenças nas mucosas (orofaríngea), tecido pulmonar e tecido urogenital das aves. Essas infecções podem ser letais em aves imunodebilitadas, incluindo embriões em desenvolvimento (Mete *et al.*, 2013; Mugale *et al.*, 2015).

Liu *et al*. (2018) enfatizaram que a candidíase em aves é comum e constitui um potencial risco à saúde para criadores e consumidores, uma vez confirmada pela análise genética a presença de *C.albicans* em humanos e aves. Já, *C. krusei* parece ter menor importância com patógeno em aves quando comparado com *C. tropicalis* e *C. parapsilosis* (Muira *et al*., 2012).

Leveduras patogênicas têm sido isoladas do conteúdo fecal e cloacal de diversas aves. Cafarchia *et al*. (2018) demonstraram a presença 19 espécies de *Candida* na cloaca, nas fezes e nos ovos de galináceos. A *Candida catenulata* e *Candida albicans* foram as espécies mais frequentes, sendo as fezes com o maior tamanho populacional, em decorrência do enriquecimento nutricional.

Estudos envolvendo a pesquisa de *Candida* spp em fezes de aves têm sido desenvolvidos em diferentes regiões do Brasil, sendo relatado o isolamento de diferentes espécies dessa levedura.

Simi *et al*. (2018) avaliaram fezes de aves, na região Central do Brasil, demonstrando a presença de *C. krusei*, *C. kefyr* e *C. famata*. Essas espécies potencialmente patogênicas nas fezes analisadas constituem um risco de exposição aos cuidadores ou outros indivíduos com contato constante, atuando como uma importante cadeia da zoones. Já, a *Candida famata* foi a única espécie encontrada nas fezes de galináceos na França (Al-Seraih *et al*., 2015).

Mendes *et al.* (2014) avaliaram a presença de leveduras potencialmente patogênicas em fezes de aves selvagens no Rio Grande do Sul, Brasil. Dentre as leveduras isoladas, foram relatadas: *Candida albicans, C. famata, C.*

guilliermondii, C. sphaerica, C. globosa, C. catenulata, C. ciferri, C. intermedia.

Frangos de corte podem apresentar enteropatógenos no trato intestinal, podendo permanecer indetectáveis antes do abate. A composição da microbiota intestinal é dependente de inúmeros fatores, incluindo o ambiente, a dieta e a antibioticoterapia (Cisek *et al*., 2014).

No presente estudo, foi observado um predomínio de bactérias nas fezes dos frangos de corte quando adultos, ocasionando em muitas situações dificuldades no isolamento de leveduras. Esses dados corroboram com outros estudos, indicando elevada presença de bactérias (95%), seguido de fungos (2%) e vírus (0,2%) nas fezes de aves (Singh *et al*., 2014).

Lee *et al*. (2017) demonstraram decréscimo na quantidade de *Candida albicans* presente na casca dos ovos de aves durante o processo de incubação e predomínio de bactérias; sugerindo como explicação às condições ótimas para o crescimento (umidade, temperatura e raios solares) que interferem na quantidade dos mesmos e consequentemente à interação entre os microrganismos da microbiota.

Dentre as 7 espécies isoladas, 100% dos isolados de *C. krusei* (4/4) e de *C. pelicullosa* (1/1) apresentaram resistência ao fluconazol. Dos isolados de *C. albicans*, 17% (1/6) apresentaram resistência ao fluconazol e itraconazol simultaneamente e 7,7% (2/26) das *C. tropicalis* apresentaram resistência a ambos antifúngicos com dose-dependência ao cetoconazol. Não houveram isolados resistentes ao anfotericina-B (Tabela 2).

Tabela 2 – Perfil de suscetibilidade aos antifúngicos avaliados dos 42 isolados de *Candida* spp.

	DROGA											
	FLUCONAZOL			**ITRACONAZOL**			**CETOCONAZOL**			**AMFOTERICINA-B**		
LEVEDURA	**S**	**SDD**	**R**	**S**	**SDD**	**R**	**S**	**SDD**	**R**	**S**	**SDD**	**R**
	N/F%			**N/F%**			**N/F%**			**N/F%**		
Candida tropicalis	**17/65**	**1/4**	**8/31**	**17/65**	**7/27**	**2/8**	**24/92**	**2/8**	**0/0**	**26/100**	**0/0**	**0/0**
Candida parapsilosis	**3/100**	**0/0**	**0/0**	**3/100**	**0/0**	**0/0**	**3/100**	**0/0**	**0/0**	**3/100**	**0/0**	**0/0**
Candida krusei	**0/0**	**0/0**	**4/100**	**2/50**	**2/50**	**0/0**	**4/100**	**0/0**	**0/0**	**4/100**	**0/0**	**0/0**
Candida albicans	**5/83**	**0/0**	**1/17**	**5/83**	**0/0**	**1/17**	**6/100**	**0/0**	**0/0**	**6/100**	**0/0**	**0/0**

Candida pelicullosa	**0/0**	**0/0**	**1/100**	**1/100**	**0/0**	**0/0**	**0/0**	**1/100**	**0/0**	**1/100**	**0/0**	**0/0**
Candida zeylanoides	**2/100**	**0/0**	**0/0**	**0/0**	**2/100**	**0/0**	**2/100**	**0/0**	**0/0**	**2/100**	**0/0**	**0/0**
Candida rugosa	**2/100**	**0/0**	**0/0**	**2/100**	**0/0**	**0/0**	**2/100**	**0/0**	**0/0**	**2/100**	**0/0**	**0/0**

C. parapsilosis, *C. albicans*, *C. tropicalis*, *C. guilliermondii*, *C. krusei* e *C. famata* foram as espécies isoladas de amostras da cloaca de emas em Fortaleza, Ceará. Todos os isolados apresentaram elevada suscetibilidade à anfotericina B, corroborando com dados do presente estudo. Brilhante *et al*. (2013) relataram índices mais elevados de resistência aos azóis. *C. albicans* (11/18), *C. parapsilosis* (2/19) e *C. tropicalis* (2/13) apresentaram resistência a dois azóis, simultameamente. Dos isolados de *C. albicans*, 83,3% e 72,2%, de *C. parapsilosis*, 36,84% e 15,79%, e de *C. tropicalis*, 46,15% e 38,46% apresentaram resistência ao fluconazol e itraconazol, respectivamente. Outro estudo desenvolvido por Brilhante *et al.* (2014) relatou isolados de *C. parapsilosis* resistentes ao fluconazol. Contrastando com os resultados do presente estudo com 17% das *C. albicans* resistentes ao fluconazol e itraconazol. Já, os isolados de *C. tropicalis* apresentaram 31% e 8% de resistência o fluconazol e itraconazol, repectivamente. Nenhum isolado de *C. parapsilosis* foi resistente aos azóis.

Lord *et al*. (2010) descreveram 9 espécies de *Candida* isoladas de amostras fecais de aves. *Candida albicans* foi a mais frequente com 28,89%, seguida da *C. krusei* (13,33%), *C. tropicalis* e *C. glabrata* (ambas com 4,44%). Nesse mesmo estudo, uma proporção significativa das leveduras isoladas demonstrou elevado perfil de resistência às drogas; mais especificamente, 18,1% foram resistentes aos 11 antifúngicos testados, 45,8% foram resistentes a 4 ou mais antifúngicos. No presente estudo, somente (2/26 amostras) de *C. tropicalis* e (1/6) de *C. albicans* apresentaram resistência ao fluconazol e itraconazol simultaneamente.

Subramanya *et al*. (2017) isolaram 8 espécies de *Candida* em fezes de aves obtidas de ambiente comercial, sendo a *C. famata*, *C. ciferrii* e *C. albicans* as espécies mais frequentes. Os isolados de levedura apresentaram elevada concentração inibitória mínima para fluconazol e anfotericina-B. A prática do uso de promotores de crescimento e antimicrobianos na ração das aves pode contribuir para o elevedo índice de resistência às drogas.

O elevado perfil de sensibilidade das *Candida* spp isoladas nas fezes dos frangos de corte no presente estudo não exime a importância de controle ambiental mais acurado, visto que o uso de biocidas para descontaminação ambiental pode ocasionar a longo prazo a emergência de resistência sob pressão seletiva. Fato esse relatado no estudo desenvolvido por Lorin *et al.* (2017) com cepas de *Aspergillus* spp e *Candida albicans.*

Reis *et al.* (2018) relataram a presença de *Candida* spp em aves na mesma região geográfica pesquisada no presente estudo. A principal espécie encontrada foi a *C. guilliermondii*, seguida de *C. famata*, *C. parapsilosis* e *C. tropicalis*. Não houve isolamento de *C. albicans*. Todas as amostras de *Candida* spp isoladas foram suscetíveis aos antifúngicos testados (cetoconazol, fluconazol, itraconazol e anfotericina-B). Resultado semelhante foi relatado no presente estudo, com baixa frequência de *C. albicans* e elevado perfil de sensibilidade aos antifúngicos testados.

Ao avaliar a frequência de isolamento de leveduras nos locais pré-determinados no aviário de criação dos frangos de corte, evidenciaram-se que as fezes coletadas próximas ao sistema de ventilação (exaustor) e porção mediana do galpão apresentaram maior contaminação quando comparadas as fezes na porção mais distante do exaustor. Roque *et al*. (2016) avaliaram a qualidade do ar de fazendas com criação de galinha que possuíam sistema de ventilação e detectaram a presença de diferentes bactérias e *Candida albicans*. Sugere-se, portanto, que o sistema de ventilação e parâmetros climáticos (umidade e temperatura) podem interferir na presença de leveduras nas fezes nos diferentes locais analisados.

A colonização da microbiota intestinal de frangos de corte pode ocorrer por transmissão horizontal no ambiente que são criados, visto que não há o contato com aves adultas, tendo o meio ambiente um forte impacto na colonização (Smith; Rehberger, 2018). Isso sugere, a presença de mesma levedura em diferentes locais do galpão de criação. Entretanto, esse padrão não foi encontrado no presente estudo, havendo grande diversidade de *Candida* spp.

CONCLUSÕES

As fezes de frango de corte apresentam espécies de *Candida* que corrobaram com dados epidemiológicos como sendo as espécies frequentes nas micoses humanas. Apesar

do presente estudo demonstrar elevada suscetibilidade aos antifúngicos que comumente são usados na prática clínica, estudos mais elaborados com análise molecular se faz necessário para uma efetiva comprovação da importância do ecossistema na interferência da saúde coletiva. Fato esse essencial para medidas preventivas com ênfase na prevenção de doenças na comunidade.

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CONCLUSÃO

Dados da literatura relataram isolamento de *Cryptococcus* spp em diferentes fontes ambientais, não sendo comprovada na presente pesquisa. Sugere-se que estressores

ambientais abióticos e bióticos tenham dificultado o isolamento, viso que a região do Noroeste Paulista apresenta maior parte do ano elevada temperatura e índices de raios solares. Dessa forma, não houve a análise filogenética entre amostras clínicas e ambientais, como se havia pretendido.

A análise filogenética entre as amostras clínicas de *Crytpococcus* spp de pacientes com HIV na região Noroeste Paulista demonstrou grande semelhante genética, corroborando com dados da literatura.

Não houve comprovação entre alteração genética e características fenotípicas na produção de melanina, em vista da pequena amostragem avaliada. Assim como também, não houve correlação do perfil filogenético com o perfil de suscetibilidade aos antifúngicos.

Os isolados de *Cryptococcus* spp em amostras clínicas apresentaram elevada sensibilidade aos antifúngicos da classe dos azóis e poliênicos. Dados semelhantes foram demonstrados em outros estudos, sugerindo melhor prognóstico aos pacientes avaliados.

Dentre as espécies de *Candida* spp isoladas em fontes ambientais, as mesmas estão em concordância com dados da literatura. Fato esse extrema relevância pois ratificam com as espécies emergentes nas doenças fúngicas humanas.

As espécies ambientais de *Candida* spp isoladas das fezes de frangos de corte apresentaram elevado perfil de sensibilidade; já, as amostras obtidas de árvores demonstraram maior percentual de resistência, havendo a necessidade imediata de maiores esforços em pesquisas para elucidar os mecanismos abióticos e bióticos envolvidos nos mecanismos de patogenicidade, assim como a ações antropogênicas.

Frente aos resultados expostos, técnicas moleculares são eficientes na investigação epidemiológico de isolados de leveduras patogênicas. Investimentos futuros para esclarecer mecanismos genéticos e fenotípicos e as implicações ambientais são necessários para compreender a história natural das patologias fúngicas e consequentemente, elaborar ações preventivas na comunidade.

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