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DÉBORA CASTELO BRANCO DE SOUZA COLLARES MAIA

RESISTÊNCIA A AZÓLICOS EM *CANDIDA* SPP. DE ORIGEM VETERINÁRIA: UM FENÔMENO MEDIADO POR BOMBAS DE EFLUXO

FORTALEZA
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Tese apresentada ao Programa de Pós-Graduação em Microbiologia Médica da Faculdade de Medicina da Universidade Federal do Ceará, como requisito parcial para a obtenção do título de doutor em Microbiologia Médica.
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Orientador: Prof. Dr. Marcos Fábio Gadelha Rocha
Co-Orientadora: Profa. Dra. Raimunda Sâmia Nogueira Brilhante

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Aos meus pais, com muito amor e gratidão.

“Keep swimming...”

Dory (Walt Disney’s Finding Nemo)

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RESUMO

O monitoramento da sensibilidade antifúngica em espécies de *Candida* de origem veterinária é uma prática recente e os mecanismos envolvidos ainda não foram completamente elucidados. Considerando que o arsenal de drogas antifúngicas é limitado e que o fenômeno de resistência tem se tornado mais freqüente, a compreensão deste fenômeno e a busca por alternativas terapêuticas se fazem necessárias. Dessa forma, o presente trabalho teve como objetivo monitorar a sensibilidade *in vitro* de *Candida* spp. oriundas de animais, com ênfase na resistência aos azólicos mediada por bombas de efluxo e na avaliação da atividade do imidazólico levamisol sobre o crescimento destas leveduras. Para tanto, em um primeiro momento, foram avaliados 126 isolados de *Candida*, sendo 19 *C. albicans*, 17 *C. famata*, 5 *C. guilliermondii*, 8 *C. krusei*, 29 *C. parapsilosis*, 48 *C. tropicalis*, dos quais 22 foram isolados de rapinantes, 32 de periquitos do sertão, 56 de papagaios, 7 de araras canindé e 3 de um ouriço-cacheiro. Todas as cepas foram submetidas ao teste de microdiluição em caldo, ante a anfotericina B, itraconazol, fluconazol, segundo metodologia padronizada pelo *Clinical Laboratory Standards Institute* (documento M27-A3). As concentrações inibitórias mínimas (CIMs) variaram de 0,03125 a 2 µg/mL, 0,125 a 250 µg/mL e de 0,03125 a 125 µg/mL para anfotericina B, fluconazol e itraconazol, respectivamente. Dos 126 isolados avaliados por microdiluição, 33 (26,2%) foram resistentes aos azólicos, sendo 7 (5,6%) resistentes somente a fluconazol, 1 (0.8%) resistente somente ao itraconazol e 24 (19%) resistentes a ambas as drogas. Em um segundo momento, todas estas cepas resistentes aos derivados azólicos, mais 20 *C. albicans* e 3 *C. tropicalis* resgatadas da nossa coleção de leveduras resistentes de origem veterinária, foram submetidas ao teste de inibição de bomba de efluxo com prometazina, perfazendo um total de 56 cepas resistentes a azólicos. Dessa forma, as CIMs de fluconazol e itraconazol sofreram reduções significativas de 2 a 250 e de 16 a 4000 vezes, respectivamente. Investigou-se, ainda, a atividade antifúngica do levamisol contra 12 *C. albicans*, 12 *C. krusei*, 12 *C. parapsilosis* e 12 *C. tropicalis*, sendo obtidas CIMs e concentrações fungicidas mínimas que variaram de 0,58 a 2,34 mg/mL e de 2,34 a 9,37 mg/mL, respectivamente. Paralelamente, foi demonstrado que o levamisol inibe a formação do biofilme de *C. albicans* e *C. tropicalis*, bem como interfere na manutenção do biofilme maduro. Os dados apontam que a resistência aos derivados azólicos é, pelo menos em parte, mediada por bombas de efluxo, bem como demonstram o potencial antifúngico do imidazólico levamisol e sua capacidade de inibir o biofilme de cepas de *Candida* spp. oriundas de animais.

Palavras-chave: Animais, *Candida* spp., azólicos, resistência, bomba de efluxo, biofilme, levamisol e prometazina.

ABSTRACT

Monitoring of the antifungal susceptibility of *Candida* species from veterinary sources is a recent practice and the mechanisms involved in antifungal resistance have not been completely elucidated. Considering that the antifungal arsenal is limited and that antifungal resistance has become more frequent, the comprehension of this phenomenon and the pursuit for therapeutic alternatives are necessary. Thus, the present work aimed at monitoring the *in vitro* susceptibility of *Candida* spp. isolated from animals, with emphasis on efflux pump-mediated azole resistance and on the evaluation of the effect of the imidazole levamisole on the growth of these yeasts. For such, in a first approach, 126 *Candida* isolates (19 *C. albicans*, 17 *C. famata*, 5 *C. guilliermondii*, 8 *C. krusei*, 29 *C. parapsilosis*, 48 *C. tropicalis*), out of which 22 were recovered from raptors, 32 from cactus parakeets, 56 from Amazon parrots, 7 from blue-and-gold macaws and 3 from a Brazilian porcupine. All isolates were submitted to broth microdilution test against amphotericin B, itraconazole and fluconazole, according to the methodology recommended by the Clinical Laboratory Standards Institute (document M27-A3). The MICs ranged from 0.03125 to 2 μg/mL, 0.125 to 250 μg/mL and 0.03125 to 125 μg/mL for amphotericin B, fluconazole and itraconazole, respectively. Out of 126 evaluated isolates, 33 (26.2%) were resistant to azoles, with 7 (5.6%) isolates resistant to fluconazole, 1 (0.8%) isolate resistant to itraconazole and 24 (19%) resistant to both drugs. In a second approach, all these azole resistant isolates, plus 20 *C. albicans* and 3 *C. tropicalis* that were recovered from our collection of resistant yeasts from veterinary sources, were submitted to the efflux pump inhibition assay with promethazine, with a total of 56 azole resistant isolates. Thus, MICs for fluconazole and itraconazole significantly reduced from 2 to 250 times for fluconazole and from 16 to 4000 times for itraconazole. The antifungal activity of levamisole against 12 *C. albicans*, 12 *C. krusei*, 12 *C. parapsilosis* and 12 *C. tropicalis*, was also evaluated, and MICs and minimum fungicidal concentrations varying from 0.58 to 2.34 mg/mL and from 2.34 to 9.37 mg/mL were obtained, respectively. Parallelly, it was demonstrated that levamisole significantly inhibits biofilm formation and interferes with the maintenance of mature biofilms. These data show that azole resistance is partially mediated by efflux-pumps and demonstrate the antifungal potential of the imidazole levamisole and its capacity of inhibiting the biofilm of strains of *Candida* spp. from animals.

Keywords: Animals, *Candida* spp., azoles, efflux pump, biofilm, levamisole and promethazine.

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ASPECTOS METODOLÓGICOS E RESULTADOS OBTIDOS COM LEVAMISOL

LISTA DE ABREVIATURAS E SIGLAS

ABC	ATP-binding cassette
AIDS	Acquired immunodeficiency syndrome
AMB	Anfotericina B
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CAS	Caspofungin
CEMM	Centro Especializado em Micologia Médica
CETAS	Centro de Triagem de Animais Silvestres
CFM	Concentração fungicida mínima
CIM	Concentração inibitória mínima
CLSI	Clinical Laboratory Standards Institute
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
FLC	Fluconazol
G	Grama
HIV	Human immunodeficiency vírus
IBAMA	Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis
ICMBio	Instituto Chico Mendes de Conservação da Biodiversidade
ITC	Itraconazol
ITIS	Integrated Taxonomic Information System
Kg	Quilograma
KTC	Cetoconazol
L	Litro
MFC	Minimum fungicidal concentration
MFS	Major Facilitators
mg	Miligrama
µg	Micrograma
MIC	Minimum inhibitory concentration
mL	Mililitro
MOPS	Ácido morfolinopropanossulfônico
Pz	Atividade de fosfolipase
RAPD	Random amplification of polymorphic DNA
rpm	Rotações por minuto
SISBIO	Sistema de Autorização e Informação em Biodiversidade
UECE	Universidade Estadual do Ceará
UFC	Universidade Federal do Ceará
VRC	Voriconazol

SUMÁRIO

1. INTRODUÇÃO

1.1 *Candida* spp. como Parte da Microbiota de Animais

As leveduras do gênero *Candida* são microrganismos eucariontes que pertencem ao Reino Fungi, filo Ascomycota; subfilo Saccharomycotina, classe Saccharomycetes e ordem Saccharomycetales (NCBI Taxonomy, 2011). O filo Ascomycota possui três grupos principais: Archiascomycetes, representado por fungos primitivos; Euascomycetes, composto por fungos filamentosos; e Hemiascomycetes, representado por leveduras. Esse último grupo abrange a grande ordem Saccharomycetales, à qual pertencem as espécies de *Candida*. Todos os membros desta ordem se caracterizam por apresentarem hifas rudimentares ou ausentes, células vegetativas que proliferam por brotamento ou fissão, parede celular com quantidade reduzida de quitina e ascos que são formados isoladamente ou em cadeia (DIEZMANN et al., 2004).

As espécies de *Candida* são componentes usuais da microbiota dos tratos gastrintestinal e geniturinário e da pele de animais saudáveis, atuando como organismos comensais (WROBEL et al., 2008; NAWANGE et al., 2009; SIMPSON et al., 2009). Muitas espécies de *Candida* já foram isoladas de vários *taxa* animais, incluindo mamíferos (BRITO et al., 2009), aves (CAFARCHIA et al., 2006; BRILHANTE et al., 2010) e animais ectotérmicos, como répteis (NARDONI et al., 2008) e crustáceos (BRILHANTE et al., 2011). No entanto, a frequência de isolamento dessas leveduras varia grandemente entre as classes animais, como será demonstrado a seguir.

O isolamento de espécies de *Candida* como parte da microbiota de mamíferos saudáveis é pouco relatado. Cleff et al. (2005) demonstraram que *Candida* spp. compõem a microbiota vaginal de cadelas sadias e que a frequência de isolamento é influenciada pela fase do ciclo reprodutivo. As espécies isoladas por esses pesquisadores foram *C. parapsilosis*, *C. guilliermondii*, *C. kefyr*, *C. albicans*, *C. glabrata* e *C. krusei*. Kobayashi (2008) demonstrou uma baixa porcentagem de isolamento de *Candida* spp. da cavidade oral de cães, obtendo somente três exemplares da espécie *C. parapsilosis*, dos 38 animais avaliados. Brito et al. (2009a), por sua vez, obtiveram 23 isolados de *Candida* spp. a partir de amostras colhidas da mucosa oral, da mucosa vaginal, do prepúcio e da região perianal de 203 cães sadios, sendo a região perianal e a vagina os sítios mais colonizados. Destes isolados, *C. parapsilosis* representou a espécies mais prevalente, seguida de *C. tropicalis* e, por último, *C. albicans*.

Além de cães, existem trabalhos relatando o isolamento de *Candida* spp. como parte da microbiota de diversos sistemas orgânicos de outros mamíferos, como cetáceos (TAKAHASHI et al., 2010; MORRIS et al., 2011), roedores (ROSTAMI et al., 2010), ruminantes (SOUZA; SIQUEIRA, 2003; SHOKRI et al. 2010; ABRÃO et al., 2011), suínos (CARREGARO et al., 2010), dentre outros. No entanto, o sistema digestório é demonstrado como aquele onde as espécies de *Candida* ocorrem com maior freqüência.

Nas aves, o isolamento de leveduras do gênero *Candida* é mais comum, quando comparado às outras classes animais, independente da ordem aviária pesquisada. Diferentemente dos mamíferos, os relatos de isolamento de *Candida* spp. em aves sadias são frequentes, principalmente, a partir de espécimes obtidos do trato gastrintestinal. Em um estudo sobre a ocorrência de leveduras em fezes de psitacídeos em cativeiro, obteve-se uma prevalência de 62% para *Candida* spp., e as espécies mais encontradas foram *C. pelliculosa*, seguida por *C. famata* (MANCIANTI et al., 2001).

Em um outro estudo, com 182 aves de rapina européias, Cafarchia et al. (2006b), isolaram *Candida* spp. de 2,7% das aves e de 9,4% das amostras de fezes avaliadas, obtendo cinco espécies distintas: *C. albicans*, *C. tropicalis*, *C. inconspicua*, *C. famata* e *C. pelliculosa*. Ainda neste estudo, 60 rapinantes foram necropsiados e amostras foram colhidas do inglúvio, do pró-ventrículo, do ventrículo e da cloaca, resultando no isolamento de *Candida* spp. em 25% das aves. A espécie mais prevalente foi *C. albicans* e os sítios anatômicos que apresentaram um maior número de isolados foram inglúvio e cloaca (CAFARCHIA et al., 2006b).

Brilhante et al. (2010), por sua vez, por meio da cultura de amostras oriundas da cavidade oral, do inglúvio e da cloaca de calopsitas (*Nymphicus hollandicus*), isolaram *Candida* spp. de 55% das aves avaliadas, considerando pelo menos um dos sítios anatômicos amostrados, e de 57,1% das amostras de fezes coletadas. A cavidade oral e o inglúvio foram os sítios onde *Candida* spp. foram mais prevalentes. Ao todo, 92 isolados de *Candida* spp., pertencentes a seis espécies (*Candida albicans, C. famata, C. glabrata, C. krusei, C. parapsilosis, C. tropicalis*), foram obtidos.

Em répteis, são poucos os trabalhos que visam à caracterização da microbiota fúngica e já foi demonstrado que os fungos filamentosos saprofíticos são mais frequentemente isolados, quando comparados às leveduras do gênero *Candida* (JACOBSON; CHEATWOOD; MAXWELL, 2000). Nardoni et al. (2008), por meio do isolamento de fungos a partir de *swabs*

de cloaca de répteis mantidos em cativeiro, obtiveram 18,5% das amostras coletadas positivas para *Candida* spp., enquanto que 92,1% das mesmas eram positivas para fungos filamentosos.

Quanto aos crustáceos, já foi demonstrado que, em culturas de camarão, as espécies de *Candida* são as leveduras mais frequentemente isoladas, tanto do trato gastrintestinal dos animais, como da água de cultivo (JOHNSON; BUENO, 2000; LEAÑO et al., 2005). Em um estudo com o camarão *Macrobrachium amazonicum*, 92,3% das leveduras isoladas pertenciam ao gênero *Candida*, sendo *C. famata* a espécie mais prevalente em camarões do cativeiro, bem como do ambiente natural (BRILHANTE et al., 2011).

Assim, é possível constatar que as leveduras do gênero *Candida* encontram-se amplamente distribuídas nos sistemas orgânicos, principalmente, no trato gastrintestinal de animais sadios, como componentes da microbiota. A frequência de isolamento de *Candida* spp. é bastante variável, devido às diferenças biológicas observadas entre os *taxa* animais, como ecologia, dieta, comportamento e habitat (CAFARCHIA et al., 2006a; CAFARCHIA et l., 2006b). Contudo, as espécies de *Candida* isoladas tendem a não variar entre hospedeiros distintos.

1.2 *Candida* spp. como Agentes Patogênicos para Animais

As leveduras do gênero *Candida*, apesar de serem microrganismos comensais, podem também ocasionar uma infecção ativa, denominada de candidíase ou candidose (BRITO et al., 2009b). No entanto, para que estas leveduras se estabeleçam como agentes infecciosos, é necessário o desequilíbrio no binômio parasita-hospedeiro (SIDRIM; ROCHA, 2004).

Dentre as alterações inerentes ao hospedeiro na medicina veterinária, os fatores predisponentes mais comuns para a ocorrência de candidíase são o imunocomprometimento, o estresse crônico, as deficiências nutricionais, o uso prolongado de glicocorticóides e de antibacterianos e a ocorrência de doenças auto-imunes e de doenças de base, como infecções virais, *Diabetes Mellitus* e câncer (VELASCO, 2000; HESELTINE et al., 2003; MORETTI et al., 2004).

A capacidade de *Candida* spp. passarem de microorganismos comensais a agentes patogênicos, sob condições hospedeiras favoráveis, depende também de vários fatores de virulência (COSTA et al., 2009), os quais contribuem para a patogenicidade dos microrganismos (ZENG et al., 2008). Dentre estes fatores, podem-se destacar a produção de enzimas hidrolíticas,

como fosfolipases e proteinases, a habilidade de aderir a células epiteliais e endoteliais e a ocorrência de alterações fenotípicas e de modulação antigênica, em decorrência da formação de pseudohifas (IBRAHIM et al., 1995; GHANNOUM, 2000; OMBRELLA; RACCA; RAMOS, 2008; ZENG et al., 2008; VIEIRA; ACQUA-COUTINHO, 2009).

Dentre esses fatores, a produção de fosfolipases merece destaque, uma vez que a elevada atividade dessas enzimas está correlacionada a outros atributos de virulência, como o aumento na capacidade de aderência às células epiteliais e na taxa de mortalidade, em modelos animais (SAMARANAYAKE et al., 2005; COSTA et al., 2009) e a maior capacidade de invasão dos tecidos hospedeiros e de formação de tubo germinativo (SAMARANAYAKE et al., 2005). Assim, as fosfolipases promovem a virulência por ocasionarem lise das células hospedeiras ou alterações nas características de superfície das mesmas, facilitando os processos de aderência e de penetração (IBRAHIM et al., 1995; SAMARANAYAKE et al., 2005).

Em 1982, Price et al. descreveram um método em placa para detectar a atividade de fosfolipase em *C. albicans*. Tal método consiste na semeadura das cepas em meio ágar Sabouraud dextrose, acrescido de gema de ovo, a qual é rica em fosfolipídios. Quando os isolados são positivos, observa-se a formação de uma zona de precipitação densa, ao redor da colônia, provavelmente, devido à formação de complexos de cálcio com os ácidos graxos, liberados em decorrência da ação enzimática sobre os fosfolipídios do meio (GHANNOUM, 2000). Dessa forma, a atividade de fosfolipase (Pz) é definida como a razão entre o diâmetro da colônia e o diâmetro total, formado pela colônia e pela zona de precipitação (GHANNOUM, 2000). Devido à facilidade de execução, a utilização do ágar gema de ovo tornou-se o método tradicional de triagem de cepas de *Candida* spp., quanto à atividade de fosfolipase (GHANNOUM, 2000), sendo o método utilizado por diversos pesquisadores (CAFARCHIA et al., 2008; OMBRELLA; RACCA; RAMOS, 2008; ZENG et al., 2008; COSTA et al., 2009, VIEIRA; ACQUA-COUTINHO, 2009, SIDRIM et al., 2010; BRILHANTE et al., 2011). No entanto, uma vez que a gema de ovo contém substrato tanto para fosfolipases, quanto para lipases, essa técnica não é específica, devendo ser utilizada somente para triagem inicial (GHANNOUM, 2000; COSTA et al., 2009).

Poucos trabalhos relatam a produção de fosfolipase por cepas de *Candida* spp. de origem animal. Cafarchia et al. (2008), demonstraram que 76,4% dos isolados de *Candida* spp. obtidos do trato gastrintestinal de rapinantes, de aves migratórias e de passeriformes produziram

fosfolipases. Vieira e Acqua-Coutinho (2009), por sua vez, observaram que 68% das cepas de isolados de *Candida* spp., oriundas do inglúvio de filhotes de papagaios (*Amazona aestiva* e *A. amazonica*) com e sem sinais clínicos de ingluvite, foram positivas para a produção dessa enzima.

Nosso grupo demonstrou que 79,7% das cepas de *Candida* isoladas do trato gastrintestinal de calopsitas produziam fosfolipases, das quais 61,7% apresentavam uma forte atividade enzimática (SIDRIM et al., 2010). Adicionalmente, também foi evidenciada a atividade dessa enzima em 12,5% dos isolados de *Candida* spp. obtidos de camarões *M. amazonicum* (BRILHANTE et al., 2011)

As infecções por *Candida* spp. geralmente acometem animais imunocomprometidos (MORETTI et al., 2000; HESELTINE et al., 2003) e relatos de candidíase são descritos em diferentes partes do mundo, acometendo diversas espécies animais, com manifestações clínicas variadas (BRITO et al., 2009b).

Nos mamíferos, os sítios anatômicos mais acometidos são pele e anexos, ouvido, tratos urinário e gastrintestinal e sistema reprodutor (HESELTINE et al., 2003; JIN; LIN, 2005; KIVARIA; NOORDHUIZEN, 2007; WROBEL et al., 2008). Em cães, as leveduras do gênero *Candida* preferem áreas constantemente úmidas, onde facilmente ocorre a maceração tecidual, como nas mucosas, nas junções mucocutâneas, nas áreas intertriginosas, nas membranas interdigitais, no canal auditivo e na face lateral da orelha (CLEFF et al., 2005). Quadros de candidíase não são comumente descritos nessa espécie animal, havendo um maior número de relatos de acometimento cutâneo (CLEFF et al., 2007; LEE et al., 2011) e de candidíases sistêmicas (GERSHENSON et al., 2011; SKORIC et al., 2011). Ademais, infecções por *Candida* já foram descritas em bovinos, ocasionando mastites (SPANEMBERG et al., 2008), em roedores, ocasionando infecções sistêmicas (NAWANGE et al., 2009; SIMPSON et al., 2009), dentre outros.

Em aves, *Candida* spp. são patógenos importantes para filhotes e para indivíduos imunocomprometidos, devido à presença de enfermidades virais, de subnutrição, de fatores estressantes, dentre outras causas (MORETTI et al., 2000; VELASCO, 2000; SAMOUR; NALDO, 2002; KLAPHAKE; CLANCY, 2005; GARCIA et al., 2007; OSORIO et al., 2007). Na maioria dos casos, a candidíase tem origem endógena, em indivíduos predispostos ao crescimento excessivo dos microorganismos da microbiota (MORETTI et al., 2000; SAMOUR; NALDO, 2002; KLAPHAKE; CLANCY, 2005). No entanto, foi observado que a doença pode

ser transmitida por meio de fômites e de sondas oro-esofágicas contaminadas (MORETTI et al., 2000; SAMOUR; NALDO, 2002).

Os psitaciformes e as aves de rapina são altamente suscetíveis a infecções por esses microrganismos, sendo o trato gastrintestinal superior o sistema mais afetado, em particular orofaringe, inglúvio e esôfago (MORETTI et al., 2000; VELASCO, 2000; SAMOUR; NALDO, 2002; GELLIS, 2006). O inglúvio é suscetível a infecções por *Candida* spp., por ser um órgão saculiforme, contendo vários nutrientes necessários para o crescimento fúngico (KANO et al., 2001).

Vale ressaltar que também há relatos de infecção por *Candida* spp. em animais ectotérmicos, como em répteis (HERNANDEZ-DIVERS, 2001; PAWAIYA et al., 2011), peixes (GATESOUPE, 2007) e camarões (LU et al., 1998).

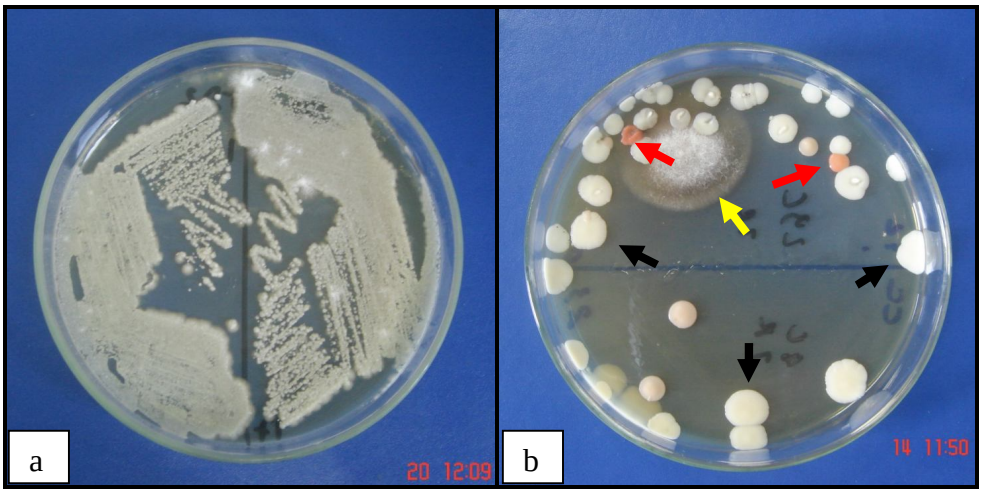
A espécie mais comumente implicada nos casos de candidíase em animais é *C. albicans*, mas outras espécies, como *C. tropicalis*, *C. parapsilosis*, *C. krusei* e *C. rugosa* tem sido comumente isoladas (MORETTI et al., 2000; VELASCO, 2000; SAMOUR; NALDO, 2002; GELLIS S., 2006; GARCIA et al., 2007; WROBEL et al., 2008). Na realidade, a prevalência de *C. albicans* em populações animais ainda não é bem conhecida (WROBEL et al., 2008).

1.3 Identificação Fenotípica de *Candida* spp.

O diagnóstico micológico de candidíase consiste, inicialmente, na confecção de lâminas contendo amostras do espécime clínico, clarificadas com hidróxido de potássio, para realização do exame direto. Posteriormente, as amostras clínicas são repicadas em meios de cultura clássicos, como ágar Sabouraud, ágar Sabouraud acrescido de cloranfenicol e ágar Sabouraud acrescido de cloranfenicol e de cicloheximida (SIDRIM; ROCHA, 2004).

Após o crescimento fúngico, as colônias são avaliadas morfologicamente, as quais são, normalmente, lisas, úmidas ou secas, de coloração branca ou creme (**Figura 1**) (DE HOOG et al., 2000). Em seguida, é realizada a avaliação micromorfológica, por meio do repique em ágar Cornmeal-Tween 80 (DE HOOG et al., 2000), a qual permite a identificação definitiva de muitas espécies de *Candida*, uma vez que forma estruturas específicas, como hifas e pseudohifas, dispostas em padrões característicos para cada espécie **(**DE HOOG et al., 2000; MILAN; ZAROR, 2004**)**. Finalmente, as cepas de *Candida* spp. são submetidas às provas bioquímicas de

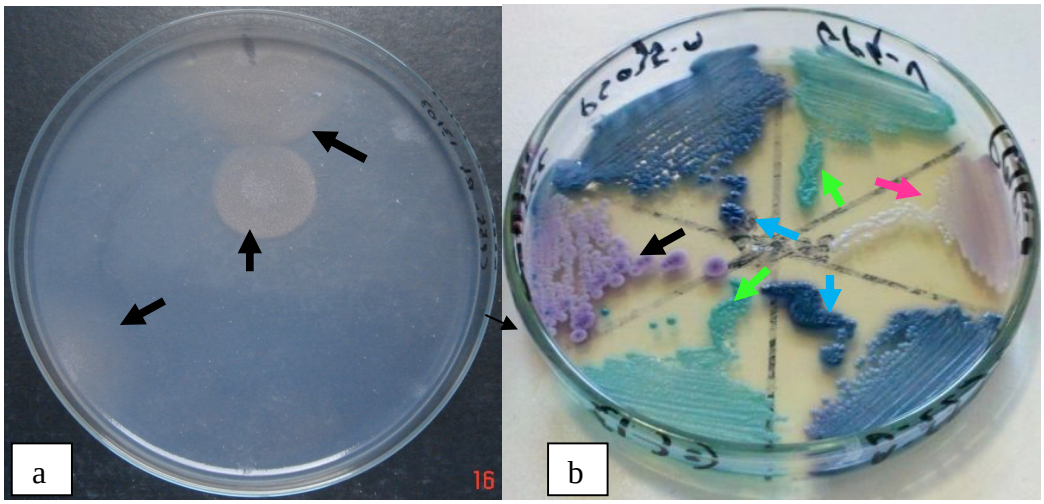
produção de urease, de assimilação e de fermentação de carboidratos e de assimilação de nitrogênio (**Figura 2**) (BRITO et al., 2009).



Fonte: CEMM, 2010 Fonte: CEMM, 2010

Figura 1: a) Placa contendo ágar semente de Níger apresentando um crescimento de múltiplas colônias coalescentes. b) Placa contendo ágar Sabouraud acrescido de cloranfenicol, demonstrando o crescimento de colônias de *Candida* spp. (setas pretas), de *Rhodotorula* spp. (setas vermelhas) e de fungos filamentosos contaminantes (seta amarela).

As características de crescimento sob temperaturas distintas também podem ser utilizadas como uma ferramenta complementar para a identificação de algumas espécies (DE HOOG et al., 2000; GARCÍA-MARTOS et al., 2004). Adicionalmente, alguns testes específicos podem ser realizados, como o crescimento em meios cromogênicos (**Figura 2**), com o objetivo de identificar colônias mistas e de realizar um diagnóstico presuntivo rápido (QUINDÓS et al., 2001) e em meios contendo concentrações distintas de cicloheximida (DE HOOG et al., 2000).



Fonte: CEMM, 2011 Fonte: CEMM, 2010

Figura 2: a) Prova de assimilação de carbono, demonstrando a presença de três halos de assimilação (setas); b) Placa contendo ágar cromogênico, permitindo a identificação presuntiva de *Candida albicans* (seta verde), *C. krusei* (seta preta) *C. parapsilosis* (seta rosa) e *C. tropicalis* (seta azul).

1.4 Resistência aos Derivados Azólicos

O arsenal terapêutico antifúngico é estreito, quando comparado ao das drogas antibacterianas, sendo composto por sete classes de drogas: 1) os derivados poliênicos, representados pela nistatina e pela anfotericina B; 2) a griseofulvina; 3) a flucitosina; 4) os derivados azólicos, como cetoconazol, fluconazol e itraconazol; 5) as alilaminas, representadas pela naftifina e terbinafina; 6) os derivados morfolínicos, representados pela amorolfina, e 7) as equinocandinas, como a caspofungina e a micafungina (ROCHA; SIDRIM, 2004). Dentre estas classes, os derivados azólicos merecem destaque por serem as drogas antifúngicas mais utilizadas na rotina clínica.

A descoberta da atividade antifúngica do benzimidazol impulsionou desenvolvimento da terapêutica antifúngica, pois, a partir desse momento, vários estudos foram conduzidos, objetivando a descoberta de derivados azólicos com propriedades antimicóticas. Assim, surgiram, inicialmente, os derivados imidazólicos, como o miconazol, o cetoconazol e o tioconazol e, posteriormente, os derivados triazólicos, representados pelo fluconazol, itraconazol, voriconazol, ravuconal e posaconazol. A descoberta das drogas triazólicas representou um grande avanço para a micologia médica, uma vez que estas drogas apresentam menos efeitos deletérios, quando comparadas aos outros antifúngicos, e propriedades farmacocinéticas mais favoráveis, resultando

em uma terapia antifúngica mais segura e eficaz (ROCHA; SIDRIM, 2004). Os derivados azólicos interferem na síntese do ergosterol por meio do bloqueio da enzima 14-α-demetilase presente no citocromo P-450 da célula fúngica, impedindo a demetilação do lanosterol em ergosterol. Esse mecanismo de ação dos azólicos altera a função da membrana celular, aumentando a permeabilidade da mesma, além de levar ao acúmulo de metabólitos tóxicos, gerados no processo de biossíntese do ergosterol (KANAFANI; PERFECT, 2008).

A resistência aos derivados azólicos começou a ser observada com o aparecimento de cepas de *C. albicans* resistentes ao fluconazol, entre pacientes HIV positivos que apresentavam candidíase oral e esofágica, previamente à introdução de terapia antirretroviral (JOHNSON, 2008; KANAFANI; PERFECT, 2008). A prevalência de *C. albicans* resistente a esse grupo de drogas é menos comum em pacientes com candidíase vaginal e candidemia. No geral, a taxa de resistência aos azólicos permanece baixa, entre a maioria das espécies de *Candida* spp. isoladas de seres humanos, variando de 1 a 2,1% em *C. albicans*, de 0,4 a 4,6% em *C. parapsilosis* e de 1,4 a 6,6% em *C. tropicalis*. (KANAFANI; PERFECT, 2008).

Existem muitos trabalhos com o monitoramento do perfil de sensibilidade a drogas de leveduras isoladas de amostras clínicas humanas. No entanto, os trabalhos com *Candida* spp. isoladas de animais não são comuns. Nosso grupo demonstrou a presença *Candida* spp. resistentes a azólicos, como parte da microbiota de animais. Assim, Brito et al. (2009) observaram que os dois isolados de *C. albicans* e três dos quatro isolados de *C. tropicalis* obtidos da cavidade oral, da região perineal, da vagina e do prepúcio de cães sadios eram resistentes a fluconazol e itraconazol. Sidrim et al., (2010), por sua vez, demonstraram que 14/39 (35,89%) isolados de *C. albicans* oriundos do trato gastrintestinal de calopsitas hígidas eram resistentes ao itraconazol e 4/39 (10,26%) eram resistentes ao fluconazol, sendo 3/39 (7,69%) resistentes a ambas as drogas. Por fim, Brilhante et al. (2011) obtiveram 8/24 (33.3%) isolados de *Candida* spp. resistentes a fluconazol e/ou itraconazol, com destaque para os isolados de *C. albicans*, os quais foram resistentes a ambas as drogas, simultaneamente.

Com base nesses achados e considerando o reduzido arsenal terapêutico antifúngico, fica clara a necessidade de realizar pesquisas que visem à busca por drogas que apresentam propriedades antimicóticas. O levamisol é um imidazólico com atividade antihelmíntica que vem sendo utilizada desde 1966 (WON et al., 2009). Esta droga que se liga a receptores colinérgicos, permitindo o influxo de cálcio para a célula muscular do parasita, o que resulta na morte do

mesmo por paralisia espástica (MARTIN; ROBERTSON, 2007). Adicionalmente, em 1978, observou-se que este composto também apresenta propriedades imuno-estimulantes, sendo bastante utilizada como adjuvante terapêutico em casos de enfermidades inflamatórias, auto-imunes e neoplásicas, como vitiligo, artrite reumatóide e câncer do colón (WON et al., 2009), e em casos de infecções associadas a quadros de imunossupressão (LAI; LU; ENG, 2002). Quanto às propriedades antimicrobianas, não há relatos sobre os efeitos inibitórios diretos do levamisol ante a *Candida* spp, no entanto, tal droga já foi utilizada como adjuvante no tratamento de candidíase oral refratária a antifúngicos convencionais, em pacientes portadores de timomas (LAI; LU; ENG, 2002).

Quanto ao desenvolvimento de resistência aos derivados azólicos em *Candida* spp., de forma geral, existem dois mecanismos primordiais. O primeiro e mais comum consiste no desenvolvimento de bombas de efluxo ativo, que são codificadas pelos genes *CDR1* e *CDR2*, os quais codificam proteínas pertencentes à superfamília ATP *binding cassette*, e pelo gene *MDR1*, que codifica uma proteína pertencente à classe *major facilitators*. A superexpressão desses genes e o consequente aumento da atividade das bombas impedem o acúmulo da droga no interior da célula, comprometendo a eficácia da mesma. A regulação positiva de *CDR1* e *CDR2* confere resistência a quase todos os azólicos, enquanto que de *MDR1*, confere resistência específica a fluconazol (KANAFANI; PERFECT, 2008). O segundo mecanismo consiste na alteração da enzima lanosterol C14-α-demetilase, molécula-alvo dos derivados azólicos, codificada pelo gene *ERG11*. A ocorrência de superexpressão ou de mutações nesse gene resulta na redução de sensibilidade ou resistência a esse grupo de antifúngicos (KANAFANI; PERFECT, 2008; MANASTIR et al., 2009).

As bombas de efluxo são divididas em duas classes principais, as proteínas ATP-*bindig cassette* (ABC) e as bombas *major facilitators* (MFS). Essas proteínas de membrana translocam ativamente compostos através das membranas celulares, utilizando diferentes fontes de energia. As proteínas ABC são transportadores primários que hidrolisam moléculas de ATP para obtenção de energia, enquanto as bombas MFS são transportadores secundários que utilizam a força gerada pela movimentação de prótons através da membrana plasmática. Os domínios de ligação de nucleotídeos das proteínas ABC e os domínios transmembrana das proteínas ABC e das bombas MFS conferem a especificidade de substrato (CANNON et al., 2009).

As proteínas ABC são encontradas em todas as células de todos os organismos, frequentemente nas membranas plasmáticas, mas também nas membranas das organelas. Algumas proteínas ABC são específicas para determinados ligantes, mas àquelas associadas à resistência a múltiplas drogas possuem uma grande variedade de substratos, como compostos hidrofóbicos, incluindo drogas. A produção elevada das proteínas Cdr1p e Cdr2p, codificadas por *CDR1* e *CDR2*, respectivamente, ocasiona a resistência aos azólicos, no geral a mais de uma droga. As bombas MFS são proteínas encontradas nas células de plantas, animais, bactérias e fungos, apresentando elevado grau de similaridade de sequência entre os diferentes reinos. A produção aumentada da proteína Mdr1p de *C. albicans*, a qual é codificada pelo gene *MDR1*, confere resistência a fluconazol nessa espécie de levedura (CANNON et al. 2009).

Diversas substâncias são capazes de modular a atividade das bombas de efluxo, como metais pesados (BRUINS et al., 2000), drogas antibacterianas, como a rifampicina (VOGEL et al., 2008), drogas citotóxicas, como a doxorrubicina (KOFLA et al., 2011) e drogas antidopaminérgicas, como as fenotiazinas (KOLACZKOWSKI et al., 2003).

As fenotiazinas são antagonistas de receptores dopaminérgicos que são utilizados clinicamente como agentes antihistamínicos ou neurolépticos para o controle de transtornos psicóticos. Já foi demonstrado que essa classe de drogas apresenta uma atividade antimicrobiana modesta, contra uma variedade de microrganismos (CHAN et al., 2007), incluindo *Candida* spp. (GALGÓCZY et al. 2011). Kolaczkowski et al. (2003) demonstraram a atividade inibitória das fenotiazinas, incluindo a prometazina, sobre a atividade das proteínas Pdr5p, Snq2p e Yor1p do fungo *Saccharomyces cerevisae*, as quais são homólogos funcionais e estruturais das proteínas transportadoras ABC de *Candida* spp. (Cdr1p e Cdr2p). Assim, por meio da adição de concentrações subinibitórias de diferentes fenotiazinas, foi possível reverter a resistência a cetoconazol em uma cepa multidroga resistente (MDR), em decorrência da produção elevada de Pdr5p. Vale ressaltar que as CIMs de cetoconazol obtidos para a cepa MDR, após a adição de uma fenotiazina, foram idênticas àquelas obtidas para cepa que sofreu deleção do gene *PDR5*, o qual codifica Pdr5p, confirmando a ação inibitória das fenotiazinas sobre a atividade das bombas ABC. Por fim, a utilização de inibidores de bombas de efluxo representa uma importante estratégia para avaliar o envolvimento dessas bombas no desenvolvimento de resistência a drogas, principalmente aos derivados azólicos (SCHUETZER-MUEHLBAUER et al., 2003).

Portanto, considerando que as leveduras do gênero *Candida* compõem a microbiota dos sistemas orgânicos de animais saudáveis e que tem sido observada a resistência primária a azólicos nesses microrganismos, torna-se importante monitorar a resistência em cepas de *Candida* de origem veterinária, visando à busca por compostos que apresentem potencial antifúngico, bem como à melhor compreensão dos principais mecanismos de resistência aos derivados azólicos.

2. PERGUNTAS DE PARTIDA E HIPÓTESES

Perguntas de Partida

1. O fenômeno de resistência primária em *Candida* spp. de origem veterinária é mediado por de bombas de efluxo?

2. A droga imidazólica levamisol apresenta atividade inibitória *in vitro* ante a *Candida* spp.?

Hipóteses

1. O mecanismo primário envolvido na resistência de *Candida* spp. de origem veterinária aos derivados azólicos é mediado por bomba de efluxo.

2. O levamisol apresenta atividade inibitória *in vitro* ante a cepas de *Candida* spp.

3. OBJETIVOS

3.1 Objetivo Geral

Com este estudo buscou-se monitorar o perfil de sensibilidade de *Candida* spp. oriundas de animais, com ênfase na resistência aos azólicos mediada por bombas de efluxo e na avaliação do potencial antifúngico do imidazólico levamisol.

3.2 Objetivos Específicos

1. Isolar e identificar espécies de *Candida* de animais silvestres mantidos em cativeiro;
2. Determinar a sensibilidade antifúngica *in vitro* a anfotericina B, itraconazol e fluconazol para as cepas isoladas;
3. Investigar o envolvimento das bombas de efluxo como mecanismo primário de resistência a azólicos em cepas de *Candida* spp. de origem veterinária;
4. Avaliar o potencial antifúngico do levamisol em cepas de *Candida* spp. de origem veterinária.

4. CAPÍTULO 1

Microbiota por leveduras de rapinantes: Uma possível ferramenta para o monitoramento ambiental

Yeast microbiota of raptors: A possible tool for environmental monitoring

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Yeast microbiota of raptors: a possible tool for environmental monitoring

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Summary

Twenty-two raptors from a rehabilitation centre were evaluated for the presence of yeasts prior to returning them to the wild, and the recovered *Candida* isolates were tested for *in vitro* antifungal susceptibility and phospholipase production. Samples were collected from the crop/lower esophagus and cloaca. *In vitro* antifungal susceptibility and phospholipase production of 21 *Candida* strains were assessed through broth microdilution and growth on egg yolk agar respectively. Twenty-seven isolates, belonging to seven species, were recovered from 16 tested birds, with *C. albicans* and *C. famata* as the most prevalent species. Three out of 21 isolates (2 *C. albicans* and 1 *C. tropicalis*) were simultaneously resistant to fluconazole and itraconazole. As for phospholipase production, 8 (8/21) isolates (6 *C. albicans*, 1 *C. famata* and 1 *C. parapsilosis*) showed enzymatic activity. The most relevant finding in this study was the isolation of resistant *Candida* spp. from wild raptors that had never been submitted to antifungal therapy, which suggests exposure to environmental contaminants. Based on this, we propose the assessment of *Candida* spp. from the gastrointestinal tract of raptors as a tool for environmental monitoring.

Introduction

Raptors belong to the avian orders *Ciconiiformes* and *Strigiformes*, which include eagles, hawks, owls and vultures (ITIS, 2011). They are commonly found in Brazilian wild animal rehabilitation centres, mainly, due to trauma-related health disorders and illegal trade (ICMBio, 2008).

According to Brazilian legislation, before releasing birds into their natural environments, they should be screened for some pathogens, including *Candida* spp. and *Cryptococcus* spp. Such procedures are routinely performed at the Triage Center for Wild Animals (CETAS) of the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA), where injured or seized wild animals are kept until they are rehabilitated and fit to be released. In this study, 22 raptors from CETAS were evaluated for the presence of yeasts, prior to returning them to the wild, and the recovered *Candida* isolates were tested for *in vitro* antifungal susceptibility and phospholipase production.

Results and discussion

Twenty-two raptors, belonging to seven different species, were assessed: 8 roadside hawks (*Rupornis magnirostris*), 6 savanna hawks (*Heterospizias meridionalis*) 4 caracaras (*Caracara plancus*), 1 American black vulture (*Coragyps atratus*), 1 yellow-headed caracara (*Milvago chimachima*), 1 Harris's hawk (*Parabuteo unicinctus*) and 1 barn owl (*Tyto alba*). The specimens *M. chimachima*, *P. unicinctus* and *T. alba* had been captured for illegal trade and were seized by IBAMA and taken to the triage centre (CETAS), where they were kept for a long period of time. The other specimens were taken from the wild to CETAS for presenting trauma-related disorders, where they spent a short period of time because they were released as soon as they recovered. While in captivity, they were maintained in collective enclosures and their diet mainly consisted of beef supplemented with minerals and vitamins. This study was approved (protocol number 02/09) by the Animal Research Ethics Committee of the Federal University of Ceará.

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Table 1. Yeast isolated from the gastrointestinal tract of raptors and *in vitro* antifungal susceptibility and phospholipase production of the recovered *Candida* spp.

Bird species	Positive /tested birds	Collection site	Yeast species	Minimum inhibitory concentration (μg ml^{-1})			Phospholipase production
				Amphotericin B	Fluconazole	Itraconazole	
Caracara plancus	3/4	Crop/lower esophagus	*T. asteroides* (1)[a]	–	–	–	–
			R. mucilaginosa (1)	–	–	–	–
		Cloaca	*C. albicans* (1)	0.5	4	0.5	0.36
			C. famata (1)	0.25	1	0.03125	1
Coragyps. atratus	1/1	Cloaca	*C. albicans* (1)	0.125	> 64[b]	> 16[b]	0.53
Heterospizias meridionalis	3/6	Crop/lower esophagus	*C. albicans* (1)	0.5	0.25	0.03125	1
			C. famata (1)	0.25	1	0.0625	0.68
			C. parapsilosis (1)	0.25	0.25	0.0625	0.97
			R. mucilaginosa (1)	–	–	–	–
		Cloaca	*C. parapsilosis* (1)	0.125	4	0.03125	1
Milvago chimachima	1/1	Cloaca	*C. albicans* (1)	0.25	1	0.03125	0.42
			C. famata (1)	–	–	–	1
Parabuteo unicinctus	1/1	Crop/lower esophagus	*C. famata* (1)	0.25	1	0.0625	1
Rupornis magnirostris	6/8	Crop/lower esophagus	*C. albicans* (3)	0.5–1	0.5–> 64[b]	0.125–> 16[b]	0.47–1
			C. famata (3)	0.125–0.5	0.5–16	0.0625–0.125	1
			C. tropicalis (2)	0.03125–0.5	1–> 64[b]	0.125–> 16[b]	1
			R. mucilaginosa (1)	–	–	–	–
		Cloaca	*C. albicans* (1)	0.5	0.5	0.03125	0.39
			C. catenulata (1)	–	–	–	1
			C. parapsilosis (1)	0.5	0.5	0.03125	1
			C. tropicalis(1)	0.25	1	0.125	1
T. alba	1/1	Cloaca	*C. famata* (1)	1	8	0.03125	1

a. Number of recovered isolates.
b. Resistant isolates.

Prior to specimen collection, the birds were clinically evaluated (Brilhante *et al.*, 2010). Only healthy birds were tested. Samples were collected from the crop/lower esophagus and cloaca and processed on birdseed (*Guizotia abyssinica*) agar supplemented with chloramphenicol (0.5 g l^{-1}) and biphenyl (0.1%), at the Specialized Medical Mycology Center of Federal University of Ceará, Brazil, as previously described (Brilhante *et al.*, 2010). Recovered colonies were identified through micromorphological and biochemical parameters. VITEK 2® (bioMérieux, USA) was used to confirm dubious identification (Brilhante *et al.*, 2010).

Sixteen out of the 22 evaluated birds (72.73%; 6/8 *R. magnirostris*, 3/6 *H. meridionalis*, 3/4 *C. plancus*, 1/1 *C. atratus*, 1/1 *M. chimachima*, 1/1 *P. unicinctus* and 1/1 *T. alba*) were positive for the presence of yeasts (Table 1). Yeasts were recovered from crop/lower esophagus, cloaca or both anatomical sites of eight (50%), five (31.25%) and three (18.75%) birds respectively.

A total of 27 isolates were obtained, with *C. albicans* ($n = 8$) and *C. famata* ($n = 8$) as the most prevalent ones, followed by *C. parapsilosis* ($n = 3$), *C. tropicalis* ($n = 3$), *Rhodotorula mucilaginosa* ($n = 3$), *C. catenulata* ($n = 1$) and *Trichosporon asteroides* ($n = 1$) (Table 1). Four individuals simultaneously presented two yeast species in the crop, which were *C. parapsilosis* and *R. mucilaginosa* from one *H. meridionalis* and *C. albicans* and *C. famata*; *C. albicans* and *C. tropicalis* or *C. famata* and *R mucilaginosa* from three *R. magnirostris*. In addition, two yeast species (*C. albicans* and *C. famata*) were simultaneously isolated from the cloaca of two birds (1 *C. plancus* and 1 *M. chimachima*) and only one bird (*R. magnirostris*) presented two yeast species in both anatomical sites (*C. famata* and *C. tropicalis* in the crop; *C. catenulata* and *C. tropicalis* in the cloaca). This last animal was the only one from which one species (*C. tropicalis*) was recovered from both evaluated sites.

Even though it is reported that the composition of the gastrointestinal yeast microbiota varies according to species-specific aspects, such as diet (Cafarchia *et al.*, 2006a), in reality, it does not seem to vary greatly, considering that the yeast species recovered in this study were similar among the evaluated bird species and to other reports concerning other avian groups, such as psittacines (Vieira and Acqua-Coutinho, 2009; Brilhante *et al.*, 2010) and ostriches (Melville *et al.*, 2004). Unlike what was expected, based on previous reports (Cafarchia *et al.*, 2006b), *Cryptococcus* spp. isolates were not recovered.

Twenty-one *Candida* isolates were submitted to antifungal susceptibility test: 8 *C. albicans* (4 from the crop/lower esophagus and 4 from the cloaca); 7 *C. famata* (5 from the crop/lower esophagus and 2 from the cloaca); 3 *C. parapsilosis* (1 from the crop/lower esophagus and 2 from the cloaca) and 3 *C. tropicalis* (2 from the crop/lower esophagus and 1 from the cloaca).

The antifungal MICs for these microorganisms were determined through broth microdilution method, as recommended by the Clinical and Laboratory Standards Institute, document M27-A3. The microdilution plates were read after 24 and 48 h of incubation at 35°C, but the MIC values considered were those obtained after 48 h of growth. MICs for fluconazole (Pfizer, Brazil) and itraconazole (Janssen Pharmaceutica, Belgium) were defined as the lowest drug concentration capable of inhibiting 50% of growth when compared with the growth control well, and for amphotericin B (Sigma Chemical Corporation, USA), it was defined as the lowest concentration at which no growth was observed. MICs of > 1, ≥ 1 and ≥ 64 μg ml^{-1} indicated resistance to amphotericin B, itraconazole and fluconazole respectively (CLSI, 2008; Sidrim *et al.*, 2010; Brilhante *et al.*, 2011). However, for *C. albicans*, *C. parapsilosis* and *C. tropicalis* the considered breakpoint for fluconazole was ≥ 8 μg ml^{-1} (Pfaller *et al.*, 2010). All strains were tested in duplicate and the assay was repeated, when resistance was detected, in order to confirm the results.

All *Candida* isolates were screened for phospholipase production, on egg yolk agar, as previously described (Price *et al.*, 1982). Phospholipase activity (Pz) was determined by calculating the ratio between the diameter of the fungal colony and the total diameter, including the colony and the precipitation zone. Pz = 1 indicated negativity for phospholipase production; Pz<1 indicated positivity for phospholipase activity and Pz<0.64 indicated strong enzymatic activity (Price *et al.*, 1982; Sidrim *et al.*, 2010; Brilhante *et al.*, 2011).

MICs for amphotericin B ranged from 0.031 to 1 μg ml^{-1} (MIC50 = 0.25 μg ml^{-1}; MIC90 = 1 μg ml^{-1}). For fluconazole and itraconazole, MICs varied from 0.125 to > 64 μg ml^{-1} (MIC50 = 1 μg ml^{-1}; MIC90 ≥ 64 μg ml^{-1}) and from 0.031 to > 16 μg ml^{-1} (MIC50 = 0.0625 μg ml^{-1}; MIC90 ≥ 16 μg ml^{-1}) respectively (Table 1). Three isolates (3/21) (two *C. albicans* and one *C. tropicalis*) exhibited *in vitro* antifungal resistance and were resistant to both azole derivatives.

Parallely, these resistant strains were tested against promethazine, which is an inhibitor of efflux pumps (Kolaczkowski *et al.*, 2003), and an MIC of 98 μg ml^{-1} was obtained for all tested isolates. After MIC determination, each isolate was tested against fluconazole and itraconazole combined with promethazine at 20 μg ml^{-1}. MICs for fluconazole dropped from > 64 to 2 μg ml^{-1} for both *C. albicans* strains and from > 64 to 16 μg ml^{-1} for the *C. tropicalis* isolate. As for itraconazole, MICs declined from > 16 to 0.0625 μg ml^{-1} for all three strains. These data suggest that the antifungal resistance observed for these *Candida* isolates was possibly associated with the overexpression of efflux pumps, since pump inhibition resulted in the decrease of azole MICs.

As for phospholipase production, out of the 23 tested *Candida* isolates, eight produced phospholipase (6 *C. albicans*, 1 *C. famata* and 1 *C. parapsilosis*). Although the sampled raptors were apparently healthy, all six positive *C. albicans* isolates presented strong enzymatic activity, with PZ values ranging from 0.36 to 0.55, similar to what was observed by Sidrim and colleagues 2010, who also isolated a high percentage of strongly positive phospholipase producing *C. albicans*. These findings corroborate those of Cafarchia and colleagues (2006b), who stated that raptors are carriers of potentially pathogenic and zoonotic yeast species, once elevated phospholipase production may be associated with enhanced virulence (Ibrahim *et al.*, 1995; Ghannoum, 2000).

Another noteworthy finding of this study was the recovery of three resistant *Candida* isolates from the crop of two *R. magnirostris* (1 *C. albicans* and 1 *C. tropicalis*) and the cloaca of one *C. atratus* (1 *C. albicans*). These three birds were free-ranging individuals from the city of Fortaleza that had recently been taken into captivity to recover from small traumas. Thus, it is known that they had never been subjected to antifungal therapy. These species are very common in urban centres, contributing to their exposure to various chemical compounds, including those from improper waste management. From 1978 to 1998, the municipal solid waste of the city of Fortaleza was destined to an open-air garbage dump, located near the Cocó River Basin. Currently, although officially deactivated, the site still receives solid waste and still contributes to environmental pollution, especially because the residues and the area do not receive proper treatment (Santos and Rigotto, 2008).

In addition, the occurrence of cross-resistance to medical and agricultural azoles in *Candida* spp. (Müller *et al.*, 2007) may also have contributed to these findings, once the two *R. magnirostris* individuals may have been exposed to azoles used in agriculture through the ingestion of small herbivore preys. However, this possibility seems less likely because Fortaleza is a big city where agriculture is not a common practice. Besides, the American black vulture (*C.atratus*) is a scavenger species (Carvalho *et al.*, 2003), thus, prey ingestion would not play an important role for the recovery of resistant yeasts.

Azole resistance has also been observed in environmental isolates of *Aspergillus* spp. (Mortensen *et al.*, 2010) and these resistant strains are also capable of infecting humans, resulting in azole-resistant cases of aspergillosis (Snelders *et al.*, 2009). Similarly, these findings concerning azole resistance in *Candida* strains from raptors also raise an important public health issue, considering that animals can represent a source of *Candida* spp. infections for humans (Edelmann *et al.*, 2005).

Historically, raptors have been used as sentinels for the presence of environmental contaminants, such as pesti-

cides and heavy metals (Marrow *et al.*, 2009). Because of their predatory behaviour, once microorganisms from the gut of preys colonize the gastrointestinal tract of raptors, the use of these birds as indicators of the presence of resistant bacteria has also been encouraged, since their microbiota reflects the environment they inhabit (Marrow *et al.*, 2009; Guenther *et al.*, 2010).

Considering the high rate of yeast isolation from animal sources, especially *Candida* spp. (Melville *et al.*, 2004; Vieira and Acqua-Coutinho, 2009; Brilhante *et al.*, 2010; 2011), these microorganisms could also provide valuable information on animals' habitats. Based on this, *Candida* spp. could be used as indicators of environmental pollution, through phenotypical assessment of their *in vitro* susceptibility profile, since azole resistance is frequently associated with the overexpression of efflux pumps (Feng *et al.*, 2010), which is possibly related to the exposure of these microorganisms to chemical compounds (e.g. pollutants), as an unspecific mechanism of cellular detoxification (Jungwirth and Kuchler, 2006). Additionally, the use of raptors for this purpose seems particularly interesting because free-ranging individuals are commonly taken to rehabilitation centres where they can easily and non-invasively be assessed, contributing to environmental monitoring.

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References

Brilhante, R.S., Castelo-Branco, D.S.C.M., Soares, G.D., Astete-Medrano, D.J., Monteiro, A.J., Cordeiro, R.A., *et al.* (2010) Characterization of the gastrointestinal yeast microbiota of cockatiels (*Nymphicus hollandicus*): a potential hazard to human health. *J Med Microbiol* **59:** 718–723.

Brilhante, R.S.N., Paiva, M.A.N., Sampaio, C.M.S., Teixeira, C.E.C., Castelo-Branco, D.S.C.M., Leite, J.J.G., *et al.* (2011) Yeasts from *Macrobrachium amazonicum*: a focus on antifungal susceptibility and virulence factors of *Candida* spp. *FEMS Microbiol Ecol* **76:** 268–277.

Cafarchia, C., Camarda, A., Romito, D., Campolo, M., Quaglia, N.C., Tullio, D., and Otranto, D. (2006a) Occurrence of yeasts in cloacae of migratory birds. *Mycopathologia* **161:** 229–234.

Cafarchia, C., Romito, D., Iatta, R., Camarda, A., Montagna, M.T., and Otranto, D. (2006b) Role of birds of prey as carriers and spreaders of *Cryptococcus neoformans* and other zoonotic yeasts. *Med Mycol* **44:** 485–492.

Carvalho, L.R., Farias, L.M., Nicoli, J.R., Silva, M.C.F., Corsino, A.T.S.M., Lima, L.A., *et al.* (2003) Dominant culturable bacterial microbiota in the digestive tract of the American black vulture (*Coragyps atratus* Bechstein 1793) and search for antagonistic substances. *Braz J Microbiol* **34:** 218–224.

Edelmann, A., Krüger, M., and Schmid, J. (2005) Genetic relationship between human and animal isolates of *Candida albicans*. *J Clin Microbiol* **43:** 6164–6166.

Feng, L., Wan, Z., Wang, X., Li, R., and Liu, W. (2010) Relationship between antifungal resistance of fluconazole resistant *Candida albicans* and mutations in ERG11 gene. *Chin Med J* **123:** 544–548.

Guenther, S., Grobbel, M., Lübke-Becker, A., Friedrich, A.G.N.D., Wieler, L.H., and Ewers, C. (2010) Antimicrobial resistance profiles of *Escherichia coli* from common European wild bird species. *Vet Microbiol* **144:** 219–225.

Ibrahim, A.S., Mirbod, F., Filler, S.G., Banno, Y., Cole, G.T., Kitajima, Y., *et al.* (1995) Evidence implicating phospholipase as a virulence factor of *Candida albicans*. *Infect Immun* **63:** 1993–1998.

Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) (2008) *Plano De Ação Nacional Para A Conservação De Aves De Rapinas*. Brasília, Brazil: ICMBio.

Integrated Taxonomic Information System (ITIS) ••. [WWW document]. URL http://www.itis.gov (Accessed on 21 June 2011).

Jungwirth, H., and Kuchler, K. (2006) Yeast ABC transporters – a tale of sex, stress, drugs and aging. *FEBS Lett* **580:** 1131–1138.

Kolaczkowski, M., Michalak, K., and Motohashi, N. (2003) Phenothiazines as potent modulators of yeast multidrug resistance. *Int J Antimicrob Ag* **22:** 279–283.

Marrow, J., Whittington, J.K., Mitchell, M., Hoyer, L.L., and Maddox, C. (2009) Prevalence and antibiotic-resistance characteristics of *Enterococcus* spp. isolated from free-living and captive raptors in central Illinois. *J Wildlife Dis* **45:** 302–313.

Melville, P.A., Cogliati, B., Mangiaterra, M.B.B.C.D., Peres, M.R., Moura, S.C.A., Matsuda, L., *et al.* (2004) Determinação da microbiota presente na cloaca e orofaringe de avestruzes (*Struthio camelus*) clinicamente sadios. *Cienc Rural* **34:** 1871–1876.

Mortensen, K.L., Mellado, E., Lass-Flörl, C., Rodriguez-Tudela, J.L., Johansen, H.K., and Arendrup, M.C. (2010) Environmental study of azole-resistant *Aspergillus fumigatus* and other Aspergilli in Austria, Denmark, and Spain. *Antimicrob Agents Chemother* **54:** 4545–4549.

Müller, F.M.C., Staudigel, A., Salvenmoser, S., Tredup, A., Miltenberger, R., and Herrmann, J.V. (2007) Cross-resistance to medical and agricultural azole drugs in yeasts from the oropharynx of human Immunodeficiency virus patients and from environmental Bavarian vine grapes. *Antimicrob Agents and Chemother* **51:** 3014–3016.

National Committee for Clinical Laboratory Standards (2008) Reference method for broth dilution antifungal susceptibility testing of yeasts: approved standard. NCCLS, document M27–MA3.

Pfaller, M.A., Andes, D., Diekema, D.J., Espinel-Ingroff, A., and Sheehan, D. (2010) Wild-type MIC distributions, epidemiological cutoff values and species-specific clinical breakpoints for fluconazole and *Candida*: time for harmonization of CLSI and EUCAST broth microdilution methods. *Drug Resist Update* **13:** 180–195.

Price, M.F., Wilkinson, I.D., and Gentry, L.O. (1982) Plate method for detection of phospholipase activity of *Candida albicans*. *Sabouraudia* **22:** 201–207.

Santos, G.O., and Rigotto, R.M. (2008) Possíveis impactos sobre o ambiente e a saúde humana decorrentes dos lixões inativos de Fortaleza (CE). *Health Environ J* **9:** 55–62.

Sidrim, J.J.C., Castelo-Branco, D.S.C.M., Brilhante, R.S.N., Soares, G.D.P., Cordeiro, R.A., Monteiro, A.J., and Rocha, M.F.G. (2010) Candida species isolated from the gastrointestinal tract of cockatiels (*Nymphicus hollandicus*): *in vitro* antifungal susceptibility profile and phospholipase activity. *Vet Microbiol* **145:** 324–328.

Snelders, E., Huis in 't Veld, R.A.G., Rijs, A.J.M.M., Kema, G.H.J., Melchers, W.J.G., and Verweij, P.E. (2009) Possible environmental origin of resistance of *Aspergillus fumigates* to medical triazoles. *Appl Environ Microbiol* **75:** 4053–4057.

Vieira, R.G., and Acqua-Coutinho, S.D. (2009) Phenotypical characterization of *Candida* spp. isolated from crop of parrots (*Amazona* spp.). *Pesqui Vet Bras* **29:** 452–456.

5. CAPÍTULO 2

Candida albicans resistente ocasionando candidemia em um ouriço-cacheiro (*Coendou prehensilis*)

Resistant *Candida albicans* causing candidemia in a wild Brazilian porcupine (*Coendou prehensilis*)

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Journal of Medical Microbiology – Case Report

Resistant *Candida albicans* causing candidemia in a wild Brazilian porcupine (*Coendou prehensilis*)

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Running title: Candidemia in a wild Brazilian Porcupine

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Summary

Systemic *Candida* infections are not commonly diagnosed among animals and candidemia caused by resistant isolates has not been reported in veterinary medicine. This case report describes the occurrence of candidemia in a wild Brazilian porcupine (*Coendou prehensilis*) caused by a multi-resistant strain of *C. albicans*. Three *Candida albicans* isolates were recovered from the lungs, lymph nodes and liver of a Brazilian porcupine, during necropsy. Afterwards, the three isolates were submitted to antifungal susceptibility analysis against amphotericin B, caspofungin, fluconazole, itraconazole, ketoconazole and voriconazole, which presented MICs of 1, 0.03125, 250, 125, 8 and 250 µg/mL, respectively. The three *C. albicans* isolates were also submitted to RAPD-PCR and M13-fingerprinting analyses, showing a genetic similarity among them, suggesting the occurrence of candidemia. This is the first report of systemic infection caused by a multi-drug resistant *C. albicans* in an animal.

Keywords: *Coendou prehensilis*, Brazilian porcupine, *Candida albicans*, antifungal resistance

Introduction

Yeasts, especially those of the genus *Candida*, are usual components of the gastrointestinal microbiota of animals, acting as commensal organisms (Wrobel *et al*., 2008; Nawange *et al*., 2009; Simpson *et al*., 2009; Brilhante *et al*., 2010, Brilhante *et al*., 2011). However, these organisms can also cause disease in humans and other animals, exhibiting a variety of clinical manifestations (Nawange *et al*., 2009), including the occurrence of systemic infections, which are not commonly described in mammals other than humans (Wrobel *et al*., 2008).

The occurrence of antifungal resistance was initially observed in *C. albicans* from AIDS patients with oropharyngeal candidiasis that did not respond to treatment with fluconazole (Kanafani & Perfect, 2008). Since then, surveillance programs of the antifungal susceptibility of *Candida* spp. isolated from human infections have been frequently performed in many countries (Pfaller & Diekema, 2007). However, surveillance of yeasts isolated from animals is not frequently performed and the prevalence of resistant *Candida* remains unknown. Additionally, even though resistant *Candida* spp. have been recovered from healthy animals (Brito *et al*., 2007, Brito *et al*., 2009, Sidrim *et al*., 2010, Brilhante *et al*., 2011), to our knowledge, systemic *Candida* infections caused by resistant isolates has not been reported. This case report describes the occurrence of candidemia in a wild Brazilian porcupine caused by a multi-resistant strain of *C. albicans*.

Case Report

An adult male Brazilian porcupine (*Coendou prehensilis*) was taken to the Triage Center for Wild Animals (CETAS) of the Brazilian Institute of Environment and Renewable Natural

Resources (IBAMA), in Fortaleza, Ceará, Brazil, for presenting fractures in both femurs, after a car hit. The animal was submitted to an osteosynthetic procedure of the left femur, followed by therapy with antibiotics (enrofloxacin 5 mg/Kg, every 24 hours, for seven days) and anti-inflammatory (ketoprofen 1 mg/Kg, every 24 hour, for three days). During recovery, the animal was kept in CETAS for two months, when it suddenly died from a generalized mixed infection with *Candida albicans*, isolated from lungs, lymph nodes and liver, and some bacterial strains (Enterobacteria and *Streptococcus hyointestinalis*) that were isolated from multiple abscesses within the thoracic and abdominal cavities.

During necropsy, samples of the organs (lung, liver, spleen, kidney and lymph nodes) that presented micro-abscesses and the content of a retroperitoneal abscess were aseptically collected with a scalpel blade and a syringe, respectively. It is important to state that the necroscopic examination was performed right after the animal's death and that during the procedure, no lesions were observed within the bowels, including ruptures or perforations.

The clinical specimens were kept in tubes containing sterile saline, until processing, when they were cultured on 2% Sabouraud dextrose agar supplemented with chloramphenicol (0.5 g/L) and 2% Sabouraud dextrose agar supplemented with chloramphenicol and cycloheximide (0.4 g/L), for fungal recovery, and on Blood agar and McConkey agar for bacterial recovery.

Yeast colonies were recovered from lung, lymph nodes and liver samples, which were identified as *C. albicans* through growth on chromogenic medium (HiCrome *Candida* Differential Agar, HiMedia, Mumbai, India), micromorphological analysis, on Cornmeal-Tween 80 agar, and biochemical features, such as urease production and sugar assimilation (Brilhante *et al.*, 2010). Bacterial isolates were recovered from micro-abscesses and retroperitoneal abscess, which were identified through VITEK 2® (bioMérieux, USA).

The three recovered *C. albicans* isolates were submitted to antifungal susceptibility analysis, according to the document M27-A3 of the Clinical Laboratory Standards Institute (CLSI, 2008). Minimum inhibitory concentrations (MICs) for amphotericin B (Sigma Chemical Corporation, USA) was the lowest concentration at which no growth was observed and for caspofungin (Merck Sharp & Dohme, Brazil), ketoconazole (Sigma Chemical Corporation, USA), fluconazole (Pfizer, Brazil), itraconazole (Janssen Pharmaceutica, Belgium) and voriconazole (Pfizer Pharmaceuticals, USA) the MIC was defined as the lowest drug concentration inhibiting 50% of growth, when compared to the growth control well (CLSI, 2008). Microdilution plates were read after 24-48 hours of incubation at 35 ^{o}C. MICs of >1, >2, >4, ≥8, ≥1 and ≥1 μg/mL indicated resistance to amphotericin B, caspofungin (CLSI, 2008), ketoconazole (Brito *et al*. 2009), fluconazole (Pfaller *et al.*, 2010), itraconazole and voriconazole (CLSI, 2008), respectively. The three *C. albicans* isolates were resistant to the four tested azole derivatives (ketoconazole MIC=8 μg/mL, fluconazole MIC=250 μg/mL, itraconazole MIC=125 μg/mL, voriconazole MIC=250 μg/mL), but not to amphotericin B (MIC=1 μg/mL) and caspofungin (MIC=0.03125 μg/mL) (Table 1).

The confirmation of the genetic similarity among the three isolates was performed through RAPD-PCR, with primer OPQ16 (5'- AGTGCAGCCA -3') (Leelayuwat *et al*. 2000), and through M13-PCR fingerprinting, with the specific sequence of the wild-type phage (5'- GAGGGTGGCGGTTCT -3') as described by Muniz *et al.* (2010). The molecular analysis through RAPD-PCR and M13-fingerprinting revealed similar band patterns among the three *C. albicans* isolates (Figure 1), indicating a genetic similarity among them.

Discussion

Candida infections are not frequently seen in domestic and wild mammals, when compared to humans (Wrobel *et al*., 2008) and birds (Velasco, 2000), with few case reports of naturally occurring systemic candidiasis (Brown *et al*., 2005; Keck *et al*., 2009; Nawange *et al*., 2009; Simpson *et al*., 2009). The three *C. albicans* isolates evaluated in the present study were recovered from internal organs that, normally, are not colonized by *Candida* species, including lymph nodes, demonstrating the occurrence of a systemic *Candida* infection. Additionally, molecular analysis with primers OPQ16 and M13 resulted in identical band patterns for the three evaluated isolates (Figure 1). Thus, considering the phenotypical and molecular similarities among these isolates, it can be stated that they are clones of the same strain, evidencing the occurrence of candidemia. According to Harriott & Noverr (2011), 27-56% of these type of infection are polymicrobial, as observed in this case, since the animal was also infected with bacterial strains (Enterobacteria and *Streptococcus hyointestinalis*) that were isolated from internal abscesses.

Overall, there are two primary mechanisms for the development of azole resistance. The first and most common mechanism of azole resistance in *Candida* spp. is associated with the overexpression of efflux pumps, encoded by the genes *CDR1* and *CDR2*, which keeps the drug from accumulating within the cell, resulting in a decreased concentration at the site of action (Kanafani and Perfect, 2008; Feng *et al*., 2010). The second mechanism involves the alteration in the enzyme lanosterol 14α-demethylase, target molecule of the azole derivatives, encoded by the gene *ERG11*. The occurrence of overexpression or genetic mutations of this gene results in the decreased susceptibility or resistance to this class of antifungals (Kanafani & Perfect, 2008; Manastir *et al*., 2009).

Considering that efflux mediated mechanisms are most commonly involved in the azole resistance of *Candida* isolates and that the isolates were recovered from a free-ranging wild mammal that had never been submitted to antifungal therapy, two hypotheses for the source of antifungal resistance can be considered. The first and most evident one is the exposure to azole antifungals used in agricultural practices, either in the wild or in captivity, since Brazilian porcupines are herbivorous animals (Roberts *et al.*, 1985) and the occurrence of cross-resistance to medical and agricultural azoles in *Candida* spp. has been reported (Müller *et al.*, 2007). The second hypothesis concerns the exposure to environmental contaminants and/or other chemical compounds, once efflux pumps are unspecific mechanisms of cellular detoxification (Jungwirth & Kuchler, 2006) that can be modulated by several substances, including heavy metals (Bruins *et al.*, 2000), antibacterial (Vogel *et al.*, 2008) and cytotoxic drugs (Kofla *et al.*, 2011).

This represents the first case of naturally occurring *Candida* systemic infection in an animal caused by a multi-drug resistant strain. These findings highlight the importance of monitoring antifungal susceptibility of *Candida* spp. from veterinary sources, especially because the affected individual was a free-ranging wild animal with no previous history of exposure to antifungals, indicating the occurrence of primary azole resistance among *Candida* spp. from veterinary sources, including wild animals.

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References

1. **Brilhante, R. S. N., Maia, D. C. B. S. C., Soares, G. D. P., Monteiro, A. J., Astete-Medrano, D. J., Cordeiro, R. A., Sidrim, J. J. C., Rocha, M. F. G. (2010).** Characterization of the gastrointestinal yeast microbiota of cockatiels (*Nymphicus hollandicus*): a potential hazard to human health. *J. Med. Microbiol.* **59**, 718–723.
2. **Brilhante, R. S. N., Paiva, M. A. N., Sampaio, C. M. S., Teixeira, C. E. C., Castelo-Branco, D. S. C. M., Leite, J. J. G., Moreira, C. A., Silva, L. P., Cordeiro, R. A., Monteiro, A. J., Sidrim, J. J. C., Rocha, M. F. G. (2011).** Yeasts from *Macrobrachium amazonicum*: a focus on antifungal susceptibility and virulence factors of *Candida* spp. *FEMS Microbiol. Ecol.* **76**, 268 - 277.
3. **Brito, E. H. S., Fontenelle, R. O. S., Brilhante, R. S. N., Cordeiro, R. A., Monteiro, A. J.; Sidrim, J. J. C., Rocha, M. F. G. (2009).** The anatomical distribution and antimicrobial susceptibility of yeast species isolated from healthy dogs. *Vet. J.* **182**, 320-326.
4. **Brito, E. H. S., Fontenelle, R. O. S., Brilhante, R. S. N., Cordeiro, R. A., Soares-Junior F. A., Monteiro, A. J., Sidrim, J. J. C., Rocha, M. F. G. (2007).** Phenotypic characterization and in vitro antifungal sensitivity of *Candida* spp. and *Malassezia pachydermatis* strains from dogs. *Vet. J.* **174**, 147–153.

5. **Brown, R. M., Thompson, C. A., Mohamed, F. M. (2005).** Systemic candidiasis in an apparently immunocompetent dog. *J. Vet. Diagn. Invest*. **17**, 272–276.
6. **Bruins, M. R., Kapil, S., Oehme, F. W. (2000).** Microbial resistance to metals in the environment. *Ecotox. Environ. Safe.* **45**, 198-207.
7. **CLSI (2008).** Clinical and Laboratory Standards Institute. Reference method for broth dilution antifungal susceptibility testing of yeasts. Aproved standart M27-A3.
8. **Feng, L., Wan, Z., Wang, X., Li, R., Liu, W. (2010).** Relationship between antifungal resistance of fluconazole resistant *Candida albicans* and mutations in *ERG11* gene. *Chin. Med. J.* **123**, 544–548.
9. **Harriott, M. M., Noverr, M. C. (2011).** Importance of *Candida*–bacterial polymicrobial biofilms in disease. *Trends Microbiol.* **19**, 557-563.
10. **Jungwirth, H., Kuchler, K. (2006).** Yeast ABC transporters – A tale of sex, stress, drugs and aging. *FEBS Lett.* **580**, 1131–1138
11. **Kanafani, Z. A., Perfect, J. R. (2008).** Resistance to antifungal agents: mechanisms and clinical impact. *Clin. Infect. Dis.* **46**, 120-128.
12. **Keck, N., Libert, C., Rispail, P., Albaric, O. (2009).** Systemic candidosis in a guanaco (*Lama guanicoe*). *Vet. Rec.* **164**, 245-246.
13. **Kofla, G., Turner, V., Schulz, B., Storch, U., Froelich, D., Rognon, B., Coste, A. T., Sanglard, D., Ruhnke, M. (2011).** Doxorubicin induces drug efflux pumps in *Candida albicans*. *Med. Mycol.* **49**, 132–142.
14. **Leelayuwat, C.; Romphruk, A.; Lulitanond, A.; Trakulsomboon, S.; Thamlikitkul, V. (2000)** Genotype analysis of *Burkholderia pseudomallei* using

randomly amplified polymorphic DNA (RAPD): indicative of genetic differences amongst environmental and clinical isolates. *Acta Tropica* **77**, 229–237.

15. **Manastir, L.; Ergon, M. C.; Yücesoy, M. (2009).** Investigation of mutations in *ERG11* gene of fluconazole resistant *Candida albicans* isolates from Turkish hospitals. *Mycoses*, **54**, 99-104.

16. **Müller, F. M. C., Staudigel, A., Salvenmoser, S., Tredup, A., Miltenberger, R., Herrmann, J. V. (2007).** Cross-Resistance to Medical and Agricultural Azole Drugs in Yeasts from the Oropharynx of Human Immunodeficiency Virus Patients and from Environmental Bavarian Vine Grapes. *Antimicrob. Agents and Chemother*. **51**, 3014-3016.

17. **Muniz, M. M., Morais e Silva Tavares, P., Meyer, W., Nosanchuk, J. D., Zancopé-Oliveira, R. M. (2010).** Comparison of different DNA-based methods for molecular typing of *Histoplasma capsulatum*. *Appl. Environ. Microbiol.* **76**, 4438-4447.

18. **Nawange, S. R., Singh, K., Naidu, J., Singh, S. M. (2009).** Naturally acquired systemic dual infection caused by *Candida famata* (*Debaryomyces hansenii*) and *Candida catenulata* in albino rats bred for sale in the market at Jabalpur (Madhya Pradesh), India. *Mycoses* **53**, 173–175.

19. **Pfaller, M. A., Andes, D., Diekema, D. J., Espinel-Ingroff, A., Sheehan, D. (2010).** Wild-type MIC distributions, epidemiological cutoff values and species-specific clinical breakpoints for fluconazole and *Candida*: Time for harmonization of CLSI and EUCAST broth microdilution methods. *Drug Resist Update* **13**, 180-195.

20. **Pfaller, M. A., Diekema, D. J (2007).** Epidemiology of invasive candidiasis, a persistent public health problem. *Clin. Microbiol. Reviews* **20**, 133-163.

21. **Roberts, M.; Brand, S.; Maliniak, E, 1985.** The biology of captive prehensile-tailed porcupines, *Coendou prehensilis*. *J. Mamm.* **66**, 476-482.

22. **Sidrim, J. J. C., Castelo-Branco, D. S. C. M., Brilhante, R. S. N., Soares, G. D. P., Cordeiro, R. A., Monteiro A. J., Rocha, M. F. G. (2010).** *Candida* species isolated from the gastrointestinal tract of cockatiels (*Nymphicus hollandicus*): *In vitro* antifungal susceptibility profile and phospholipase activity. *Vet. Microbiol*. **145**, 324–328.

23. **Simpson, V. R., Davison, N. J., Borman, A. M., Linton, C. J., Everest, D. (2009).** Fatal candidiasis in a wild red squirrel (*Sciurus vulgaris*). *Vet. Rec.* **164**, 342-344.

24. **Velasco, M. C. (2000).** Candidiasis and cryptococcosis in birds. *Semin. Avian Exot. Pet Med*. **9**, 75-81.

25. **Vogel, M., Hartmann, T., Koberle, M., Treiber, M., Autenrieth, I. B., Schumacher, U. K. (2008).** Rifampicin induces *MDR1* expression in *Candida albicans*. *J. Antimicrob. Chemother*. **61**, 541–547

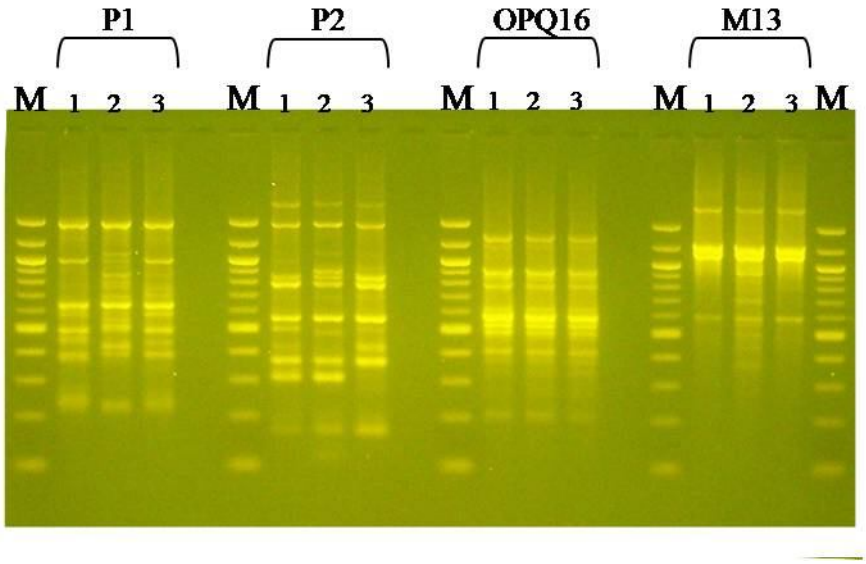
26. **Wrobel, L., Whittington, J. K., Pujol, C., Oh, S. H., Ruiz, M. O., Pfaller, M. A., Diekema, D. J., Soll, D. R., Hoyer, L. L. (2008).** Molecular Phylogenetic Analysis of a Geographically and Temporally Matched Set of Candida albicans Isolates from Humans and Nonmigratory Wildlife in Central Illinois. *Eukaryotic Cell* **7**, 1475–1486.

Table I: Minimum inhibitory concentrations of antifungals against three isolates of *Candida albicans* from a Brazilian Porcupine

***C. albicans* isolate**	**Minimum Inhibitory Concentrations (µg/mL)**					
	AMB	CAS	KTC	FLC	ITC	VRC
Lung	1	0.03125	8	250	125	250
Lymph Nodes	1	0.03125	8	250	125	250
Liver	1	0.03125	8	250	125	250

AMB: amphotericin B, CAS: caspofungin, KTC: ketoconazole, FLC: fluconazole, ITC: itraconazole, VRC: voriconazole

Figure 1: RAPD-PCR and M13-fingerprinting of the three *Candida albicans* isolates from a Brazilian Porcupine revealing similar band patterns among them. M: 100 bp molecular marker; P1, P2, OPQ16 and M13: primers used; 1: *C. albicans* recovered from lungs; 2: *C. albicans* recovered from lymph nodes; 3: *C. albicans* recovered from liver.



6. CAPÍTULO 3

Resistência a azólicos mediada por bomba de efluxo em isolados *Candida* oriundos de animais

Efflux-mediated azole resistance in *Candida* isolates recovered from animals

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Efflux-mediated azole resistance in *Candida* isolates recovered from animals

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Running title: Efflux-mediated resistance in *Candida* from animals

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Abstract

This study aimed at monitoring the *in vitro* antifungal susceptibility of *Candida* spp. isolated from wild-born Brazilian psittacines and at investigating the role of efflux pumps in the occurrence of azole resistance. Initially, clinical specimens from oral cavity, crop and cloaca were collected from 59 wild-born Brazilian psittacines, and the material was processed for the isolation of *Candida* spp, which resulted in the recovery of 101 isolates. These isolates (8 *C. albicans*; 10 *C. famata*; 4 *C. guilliermondii*; 8 *C. krusei*; 26 *C. parapsilosis*; 45 *C. tropicalis*) were submitted to antifungal susceptibility analysis against amphotericin B, fluconazole and itraconazole, obtaining MICs that ranged from 0.0625 to 2 μg/mL, from 0.25 to 250 μg/mL and from 0.03125 to 125 μg/mL, respectively. Out of these 101 isolates, 27 were resistant to azole derivatives. Afterwards, all these azole resistant isolates, plus 20 *C. albicans* and 3 *C. tropicalis* that were recovered from our collection of resistant yeasts from veterinary sources, were submitted to the efflux pump inhibition assay with promethazine, with a total of 50 azole resistant isolates. Statistically significant MIC reductions were observed for both fluconazole and itraconazole, with MICs ranging from 0.25 to 32 μg/mL and from 0.03125 to 0.25 μg/mL, respectively. Promethazine caused a greater reduction in the MICs for itraconazole when compared to fluconazole. These findings show that the increased activity of ABC efflux pumps partly represents the mechanism for azole resistance in *Candida* isolates from animals.

Keywords: *Candida* spp., azole resistance, promethazine, efflux-pump mediated resistance.

1. Introduction

Candida species are usual components of the gastrointestinal microbiota of animals, acting as commensal organisms [1-3]. Azole resistance in isolates from human beings has been reported since the beginning of the AIDS era [4] and different researches have been developed to elucidate the resistance mechanisms manifested by *Candida* spp. isolated from human clinical cases [5-7]. On the other hand, surveillance of the antifungal susceptibility of *Candida* spp. isolated from animals is not frequently performed and the prevalence of resistant *Candida* and their main resistance mechanisms remain unknown.

The most common mechanism of azole resistance in *Candida* spp. is associated with the overexpression of ATP-binding cassette (ABC) efflux pumps, mainly Cdr1 and Cdr2, which keeps the drug from accumulating within the cell, resulting in a decreased drug concentration at the site of action [4]. Efflux pump inhibitors have been used to study the role of these pumps in the development of drug resistance [8,9] and phenothiazines have shown to be potent modulators of yeast multidrug resistance, through the inhibition of Cdr1 and Cdr2 [10].

Thus, this study aimed at monitoring the *in vitro* antifungal susceptibility of *Candida* spp. isolated from animals and at investigating the role of efflux pumps in the occurrence of azole resistance.

2. Materials and Methods

2.1 Evaluated *Candida* isolates

One-hundred twenty-four *Candida* isolates were submitted to antifungal susceptibility analysis, out of which 101 were isolated from wild-born captive psittacines and 23 were recovered from the culture collection of the Specialized Medical Mycology Center (CEMM) of

the Federal University of Ceará. These 23 isolates were obtained from dogs (2 *C. albicans*; 2 *C. tropicalis*) [11]; cockatiels (16 *C. albicans*) [12] and *Macrobrachium amazonicum* prawns (2 *C. albicans*; 1 *C. tropicalis*) [3].

2.2 Animals

Fifty-nine apparently healthy captive psittacines, out of which 22 were cactus parakeets (*Aratinga cactorum*), 26 blue-fronted Amazon parrots (*Amazona aestiva*), 6 orange-winged Amazon parrots (*A. amazonica*) and 5 blue-and-gold macaws (*Ara ararauna*), were submitted to clinical evaluation and sample collection. These birds were maintained at the Triage Center for Wild Animals (CETAS) of the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA). They were all wild-born individuals that had been captured for illegal trade and were seized by IBAMA and taken to the triage center (CETAS), where they were kept for a long period of time, until they were destined to zoos or fit to be released into the wild. While at CETAS, they were maintained in collective enclosures and their diet mainly consisted of fruits and seeds. This study was approved by the System of Authorization and Information in Biodiversity (SISBIO) of the Chico Mendes Institute for the Conservation of Biodiversity (ICMBio, authorization number 23591-1) and by the Ethics Committee of Animal Research of the Federal University of Ceará (protocol number 02/09).

2.3 Sample collection and Microbiological Processing

Samples were collected from oral cavity and cloaca with sterile cotton swabs and from crop, through a ravage with sterile saline [2]. All samples were processed at the Specialized Medical Mycology Center of the Federal University of Ceará, Brazil, where they were cultured

on birdseed (*Guizotia abyssinica*) agar, supplemented with chloramphenicol (0.5 g/L) and biphenyl (0.1%) [2].The cultured media were incubated at 25 ºC, for 10 days, and were daily observed. Recovered colonies were identified based on growth on chromogenic medium (HiCrome Candida Differential Agar, HiMedia, Mumbai, India), microscopic morphology on Cornmeal-Tween 80 agar and biochemical parameters (urea hydrolysis and sugar assimilation) [2,9].

2.4 Antifungal Susceptibility Testing

One-hundred one *Candida* isolates recovered from psittacines, as described above, were monitored for the occurrence of antifungal resistance. In addition, 23 azole resistant *Candida* isolates from the culture collection of CEMM were re-tested in order to confirm the minimum inhibitory concentrations that were previously obtained for fluconazole and itraconazole. The 101 recovered *Candida* isolates (8 *C. albicans*; 10 *C. famata*; 4 *C. guilliermondii*; 8 *C. krusei*; 26 *C. parapsilosis*; 45 *C. tropicalis*) were submitted to antifungal susceptibility analysis. Minimum inhibitory concentrations (MICs) for amphotericin B (Sigma Chemical Corporation, USA), fluconazole (Pfizer, Brazil) and itraconazole (Janssen Pharmaceutica, Belgium) were determined through a broth microdilution method, according to the document M27-A3 of the Clinical Laboratory Standards Institute [13]. The concentration range tested for amphotericin B was from 0.03125 to 16 µg/mL, while for fluconazole it ranged from 0.125 to 1000 µg/mL and for itraconazole from 0.03125 to 250 µg/mL.

Susceptibility testing was performed on 96-well microdilution trays, by using RPMI 1640 medium, with L-glutamine (HiMedia, Mumbai, India), buffered to pH 7 with 0.165M morpholinepropanesulfonic acid (MOPS). The final concentration of the fungal inocula ranged

from 0.5 to 2.5 x 10^3 cells/mL [13]. The plates were incubated at 35 ^{o}C, for 48 hours. For each isolate, drug-free and yeast-free controls were included and all isolates were tested in duplicate. For amphotericin B, the MIC was the lowest concentration at which no growth was observed and for azole derivatives, the MIC was defined as the lowest drug concentration inhibiting 50% of growth, when compared to the growth control well [13].

As quality control for each test performed, *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were included. MICs of >1, ≥64 and ≥1 μg/mL were considered resistant to amphotericin B, fluconazole and itraconazole [13], respectively. However, for *C. albicans*, *C. parapsilosis* and *C. tropicalis* the considered breakpoint for fluconazole was ≥8 μg/mL [14]. All strains were tested in duplicate and the assay was repeated, when resistance was detected, in order to confirm the results.

2.5 Analysis of Efflux Pump Activity

In order to evaluate the role of efflux pump activity in azole resistant *Candida* isolates from animals, the phenotypical assay of pump modulation was performed according to Kolaczkowski et al. [10], with some modifications. Fifty strains were submitted to this assay: 23 (20 *C. albicans* and 3 *C. tropicalis*) from the culture collection of CEMM and 27 from the psittacine birds (4 *C. albicans*; 2 *C. parapsilosis* and 21 *C. tropicalis*). Briefly, the 50 resistant strains were initially tested against promethazine, which is an inhibitor of efflux pumps [10], and MICs ranging from 49 to 98 μg/mL were obtained for all tested isolates. Afterwards, a sub-inhibitory concentration of promethazine (12 μg/mL) was incorporated to the final inoculum of each tested isolate and the antifungal susceptibility analysis was performed, as previously

described, through broth microdilution, against fluconazole and itraconazole at concentrations ranging from 0.125 to 64 μg/mL and from 0.03125 to 16, respectively.

2.6 Statistical Analysis

Student's t-test for paired samples was performed to evaluate MIC reductions for fluconazole and itraconazole. A significance level of 5% was adopted for significant conclusions ($P<0.05$). In addition, a linear regression model was built, considering the MIC for the antifungal alone as the independent variable and the antifungal MIC, after combination with promethazine, as the dependent variable.

3 Results

One-hundred one *Candida* isolates were recovered from 22 cactus parakeets, 26 blue-fronted Amazon parrots, 6 orange-winged Amazon parrots and 5 blue-and-gold macaws that were kept in captivity after being seized by IBAMA. The oral cavity was the anatomical site with the highest number of isolates (54/101, 53.5%), followed by the crop (26/101, 25.7%) and the cloaca (21/101, 20.8%). Considering the isolated species, *C. tropicalis* was the most prevalent one, with 45 isolates (44.6%), followed by *C. parapsilosis* (26/101, 25.7%), *C. famata* (10/101, 9.9%), *C. albicans* and *C. krusei* (8/101, 7.9%) and *C. guilliermondii* (4/101, 4%) (Table 1). Twelve individuals presented more than one *Candida* species in the oral cavity, with the simultaneous isolation of *C. parapsilosis* and *C. tropicalis* from two cactus parakeets and three blue-fronted parrots, *C. famata* and *C. parapsilosis*, *C. famata* and *C. tropicalis*, *C. guilliermondii* and *C. krusei*, and *C. krusei* and *C. tropicalis* from one, three, one and one blue-fronted parrot, respectively, and *C. krusei*, *C. parapsilosis* and *C. tropicalis* from one blue-

fronted parrot. Considering the crop, *C. krusei* and *C. parapsilosis* were simultaneously isolated from a blue-fronted parrot and *C. krusei* and *C. tropicalis* were isolated from two macaws. Finally, *C. parapsilosis* and *C. tropicalis* were simultaneously isolated from the cloaca of one parakeet, one macaw and one blue-fronted parrot.

All recovered isolates were submitted to the antifungal susceptibility assay. The MICs obtained for amphotericin B ranged from 0.0625 to 2 μg/mL, demonstrating no resistant isolates, and for fluconazole and itraconazole the values ranged from 0.25 to 250 μg/mL and from 0.03125 to 125 μg/mL, respectively. A total of 27 (26.7%) isolates were resistant to azoles, out of which 4 were *C. albicans* (2 from the oral cavity and 1 from the crop of macaws and 1 from the oral cavity of a blue-fronted parrot), 2 *C. parapsilosis* (1 from the oral cavity of a cactus parakeet and 1 from the cloaca of a blue-fronted parrot) and 21 *C. tropicalis* (4 from the oral cavity, 3 from the crop and 6 from the cloaca of cactus parakeets, 2 from the oral cavity of blue-fronted parrots, 2 from oral cavity and 1 from the crop of orange-winged parrots and 2 from the crop and 1 from the cloaca of macaws) (Table 1). Out of these 27 isolates, 19 were resistant to both azoles, seven were resistant to fluconazole only and one was resistant to itraconazole. As for the 23 strains recovered from the culture collection of CEMM, the MIC for fluconazole ranged from 1 to 250 μg/mL and for itraconazole from 0.03125 to 64 μg/mL, confirming the occurrence of azole resistance. Thus, a total of 50 isolates (24 *C. albicans*, 2 *C. parapsilosis* and 24 *C. tropicalis*) were azole resistant, out of which 35 were resistant to both azoles, 10 were resistant to fluconazole and five were resistant to itraconazole.

Overall, the addition of promethazine significantly reduced the MICs for fluconazole ($P<0.05$) and itraconazole ($P<0.05$), which presented MIC ranges of 0.25 to 32 μg/mL and 0.03125 to 0.25 μg/mL, respectively. The linear regression analysis showed that the MIC

reduction occurs, in an independent manner, towards values close to the mean. Thus, strains with higher initial MICs suffer a more accentuated reduction, than those with lower initial MIC values. This pattern was stronger for fluconazole, when compared to itraconazole (Table 2).

Promethazine caused a greater reduction in the MICs for itraconazole when compared to fluconazole ($P<0.05$). Out of the 45 isolates that were resistant to fluconazole, five (11.1%) did not suffer any MIC alteration and 40 (88.9%) were sensitized to this antifungal, after the addition of promethazine, but only 21 (46.7%) suffered reversion of the resistance phenotype. On the other hand, all 40 (100%) itraconazole resistant isolates were sensitized to this drug, when promethazine was added, and the reversal of the resistance phenotype was observed for all them.

4 Discussion

Candida species are commensal microorganisms of the gastrointestinal tract of animals [1-3,11], presenting a particularly high isolation rate from birds [1,2,15]. In this study, 101 isolates belonging to six species were recovered from four psittacine species. These findings show that the composition of the yeast gastrointestinal microbiota does not seem to vary greatly among different bird species, once the isolated *Candida* spp. in this study were similar to those from other studies with different avian groups [15].

Considering the antifungal susceptibility analysis, even though the adopted interpretive breakpoint for amphotericin B in this study was >1 µg/mL, none of the tested *Candida* isolates were considered to be resistant to amphotericin B, once the MIC values for this drug were 2 µg/mL only against the isolated *C. krusei*, which is known for presenting decreased susceptibility to this antifungal [4]. Similarly, fluconazole MICs were higher against all C. *krusei*, also as expected for this *Candida* species [4]. Therefore, 27 (26.7%) isolates were considered to be

resistant to the tested azole drugs. This resistance rate was similar to those reported for *Candida* isolates from healthy animal sources, as dogs (22.7%) [11], cockatiels (25.4%) [12] and prawns (33.3%) (Brilhante et al., 2011), but higher than those reported for humans (6.2%) [16].

The most common mechanism of azole resistance in *Candida* spp. is associated with the increased activity of the ABC (Cdr1 and Cdr2) and MFS (Mdr1) efflux pumps, which keeps the drug from accumulating within the cell, resulting in a decreased concentration at the site of action [4]. Special attention must be given to Cdr1 and Cdr2 because they are more frequently involved, promoting cross-resistance among azoles, while Mdr1 only mediates fluconazole resistance [6,17]. Therefore, during this research, investigating the expression of these pumps was prioritized in order to evaluate the predominant azole resistance mechanism among *Candida* from animals.

Efflux pump inhibitors have been successfully used to evaluate the role of efflux pumps in the development of drug resistance [8,9]. Phenothiazines inhibit the transport of calcium, by preventing its binding to calcium-binding proteins, inhibiting calcium-dependent enzyme systems, such as those involved in the hydrolysis of ATP [18]. Considering that ATP-binding cassette (ABC) efflux pumps actively translocate compounds, including drugs, across cell membranes, using the hydrolysis of ATP as source of energy [17], phenothiazines, including promethazine, inhibit the activity of these efflux pumps [10].

Fifty isolates were submitted to the analysis efflux pump activity, through the phenotypical assay of pump modulation. Out of these 50 isolates, 27 were recovered from wild-born psittacines and 23 were recovered from the culture collection of CEMM. Overall, the addition of promethazine significantly reduced the MICs for both azole drugs. Fluconazole MICs suffered 2-to-250-fold reductions, while those for itraconazole reduced 16 to 4000 times. It was

observed that for both drugs the MICs decreased to a common level, independently of the initial values, thus, strains with higher initial MICs suffered more accentuated reductions. This observation suggest that promethazine itself, at the concentrations used, does not inhibit fungal growth, however, it acts re-establishing the susceptibility to azole drugs, once the reduced MICs tend to fall within the normal range for each drug.

The reversion of the resistance phenotype, after the addition of promethazine, indicates that resistance is mediated by ABC pump activity. In this research, it was observed that resistance to itraconazole among *Candida* isolates from animals was exclusively associated with the increased activity of these pumps, independently of the animal source (wild psittacines, dogs, cockatiels and prawns), as the addition of promethazine reversed resistance in all tested isolates. For fluconazole, on the other hand, considering that the addition of promethazine did not sensitize all tested isolates and only reversed 46.7% of the fluconazole resistant isolates, it seems that resistance to this azole drug, among *Candida* spp. from animals, presents a multifactorial nature, involving mechanisms other than the increased activity of ABC efflux pumps, such as the increased activity of the major facilitator (MFS) efflux pumps, through the overexpression of *MDR1*, or up-regulation or point mutations of the gene *ERG11* [19].

Interestingly, out of the 24 fluconazole resistant isolates that did not become susceptible after the addition of promethazine, 20 were *C. tropicalis* from parakeets (n=11), blue-fronted parrots (n=2), orange-winged parrots (n=3), macaws (n=3) and wild-harvested *M. amazonicum* prawns (n=1) and four were *C. albicans* from cockatiels (n=3) and wild-harvested *M. amazonicum* prawns (n=1). Additionally, out of the 24 fluconazole resistant *C. tropicalis*, only four from parakeets (n=2) and dogs (n=2) became susceptible to this azole drug. Based on these findings, it seems like fluconazole resistance in *C. tropicalis* is not primarily mediated by ABC

efflux pumps, corroborating the findings of Vandeputte et al. [7], who demonstrated that the multi-drug azole resistance of a clinical isolate of *C. tropicalis* was a possible consequence of a point mutation in the *ERG11* gene.

Considering the animal species, Cdr1/Cdr2 mediated itraconazole resistance was observed in *Candida* isolates from all evaluated animals, while for fluconazole resistance, this mechanism was primarily involved in isolates from parakeets (1 *C. parapsilosis*, 2 *C. tropicalis*), parrots (1 *C. albicans*, 1 *C. parapsilosis*), macaws (2 *C. albicans*), dogs (2 *C. albicans*, 2 *C. tropicalis*), cockatiels (9 *C. albicans*) and *M. amazonicum* prawns (1 *C. albicans*).

As for the source of azole resistance, some hypothesis can be considered. For those isolates recovered from birds (wild psittacines and cockatiels), considering that none of them had been submitted to antifungal therapy, we strongly believe that the occurrence of azole resistance is associated with the exposure to azole antifungals commonly used in agricultural practices, once the evaluated bird species mainly feed on seeds and fruits and the occurrence of cross-resistance to medical and agricultural azoles in *Candida* spp. has been reported [20].

Concerning the isolates recovered from dogs, azole resistance may be associated with previous antifungal therapy, even though the sampled animals had not been treated with antifungals until six months before the collection of clinical specimens [11]. In addition, the ingestion of food preservatives that are used to conserve industrialized dog food might be related to azole resistance in commensal *Candida* species, as it has been demonstrated that food spoilage yeasts develop ABC efflux pump-mediated resistance to weak acids that are used as preservatives [21].

Finally, considering that efflux pumps are unspecific mechanisms of cellular detoxification [22] that can be modulated by several substances, including common pollutants,

such as heavy metals [23], we believe that azole resistance in *Candida* from wild-harvested *M. amazonicum* occurs as a response to the exposure to environmental contaminants that are in the water of the lake where these prawns inhabited [3].

These findings suggest that *Candida* spp. recovered from animals present a higher azole resistance rate when compared to those from humans and that the increased activity of ABC efflux pumps represents the primary mechanism for azole resistance in these *Candida* isolates. Additionally, considering that animals can represent a source of *Candida* infections for humans [1,24], it is important to monitor the antifungal susceptibility of *Candida* from veterinary sources.

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5 References

[1] Wrobel L, Whittington JK, Pujol C, Oh SH, Ruiz MO, Pfaller MA, Diekema DJ, Soll DR, Hoyer LL. Molecular Phylogenetic Analysis of a Geographically and Temporally Matched Set of Candida albicans Isolates from Humans and Nonmigratory Wildlife in Central Illinois. Eukaryotic Cell 2008; 7: 1475–1486.

[2] Brilhante RSN, Maia DCBSC, Soares GDP, Monteiro AJ, Astete-Medrano DJ, Cordeiro RA, Sidrim JJC, Rocha MFG. Characterization of the gastrointestinal yeast microbiota of cockatiels (*Nymphicus hollandicus*): a potential hazard to human health. J Med Microbiol 2010; 59: 718–723.

[3] Brilhante RS, Paiva MAN, Sampaio CMS, Teixeira CEC, Castelo-Branco DSCM, Leite JJG, Moreira CA, Silva LP, Cordeiro RA, Monteiro AJ, Sidrim JJC, Rocha MFG. Yeasts from *Macrobrachium amazonicum*: a focus on antifungal susceptibility and virulence factors of *Candida* spp. FEMS Microbiol Ecol 2011; 76: 268 - 277.

[4] Kanafani ZA, Perfect JR. Resistance to antifungal agents: mechanisms and clinical impact. Clin Infect Dis 2008; 46: 120-128.

[5] Perea S, Lopez-Ribot JL, Kirkpatrick WR, Mcatee RK, Santillan RA, Martinez M, Calabrese D, Sanglard D, Patterson TF. Prevalence of Molecular Mechanisms of Resistance to Azole Antifungal Agents in *Candida albicans* Strains Displaying High-Level Fluconazole Resistance Isolated from Human Immunodeficiency Virus-Infected Patients. Antimicrob Agents Chemother 2001; 45: 2676–2684.

[6] Schuetzer-Muehlbauer M, Willinger B, Egner R, Ecker G, Kuchler K. Reversal of antifungal resistance mediated by ABC efflux pumps from *Candida albicans* functionally expressed in yeast. Int J Antimicrob Agents 2003; 22: 291-300.

[7] Vandeputte P, Larcher G, Bergès T, Renier G, Chabasse D, Bouchara JP. Mechanisms of azole resistance in a clinical isolate of *Candida tropicalis*. Antimicrob Agents Chemother 2005; 49: 4608-4615.

[8] Kriengkauykiat J, Porter E, Lomovskaya O, Wong-Beringer A. Use of an Efflux Pump Inhibitor to Determine the Prevalence of Efflux Pump-Mediated Fluoroquinolone Resistance and Multidrug Resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother 2005; 49: 565–570.

[9] Tohidpour A, Peerayeh SN, Mehrabadi JF, Yazdi HR. Determination of the efflux pump-mediated resistance prevalence in pseudomonas aeruginosa, using an efflux pump inhibitor. Curr Microbiol 2009; 59: 352–355.

[10] Kolaczkowski M, Michalak K, Motohashi N. Phenothiazines as potent modulators of yeast multidrug resistance. Int J Antimicrob Ag 2003; 22: 279-283.

[11] Brito EHS, Fontenelle ROS, Brilhante RSN, Cordeiro RA, Monteiro AJ, Sidrim JJC, Rocha MFG. The anatomical distribution and antimicrobial susceptibility of yeast species isolated from healthy dogs. Vet J 2009; 182: 320-326.

[12] Sidrim JJC, Castelo-Branco DSCM, Brilhante RSN, Soares GDP, Cordeiro RA, Monteiro AJ, Rocha MFG. *Candida* species isolated from the gastrointestinal tract of cockatiels (*Nymphicus hollandicus*): *In vitro* antifungal susceptibility profile and phospholipase activity. Vet Microbiol 2010; 145: 324–328.

[13] CLSI, 2008. Clinical and Laboratory Standards Institute. Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard M27-A3.

[14] Pfaller MA, Andes D, Diekema DJ, Espinel-Ingroff A, Sheehan D. Wild-type MIC distributions, epidemiological cutoff values and species-specific clinical breakpoints for fluconazole and *Candida*: Time for harmonization of CLSI and EUCAST broth microdilution methods. Drug Resist Update 2010; 13: 180-195.

[15] Cafarchia C, Camarda A, Romito D, Campolo M, Quaglia NC, Tullio D, Otranto D. Occurrence of yeasts in cloacae of migratory birds. Mycopathol 2006; 161: 229-234.

[16] Pfaller MA, Diekema DJ, Gibbs DL, Newell VA, Ellis D, Tullio V, Rodloff A, Fu W, Ling TA. The Global Antifungal Surveillance Group. Results from the

ARTEMIS DISK Global Antifungal Surveillance Study, 1997 to 2007: a 10.5-Year analysis of susceptibilities of *Candida* species to fluconazole and voriconazole as determined by CLSI Standardized disk diffusion. J Clin Microbiol 2010; 48: 1366–1377.

[17] Cannon RD, Lamping E, Holmes AR, Niimi K, Baret PV, Keniya MV, Tanabe K, Niimi M, Goffeau A, Monk BC. Efflux-mediated antifungal drug resistance. Clin Microbiol Rev 2009; 22: 291-321.

[18] Martins M, Dastidar SG, Fanning S, Kristiansen JE, Molnar J, Pages JM, Schelz Z, Spengler G, Viveiros M, Amaral L. Potential role of non-antibiotics (helper compounds) in the treatment of multidrug-resistant Gram-negative infections: mechanisms for their direct and indirect activities. Int J Antimicrob Ag 2008; 31: 198–208.

[19] Siikala E, Rautemaa R, Richardson M, Saxen H, Bowyer P, Sanglard D. Persistent *Candida albicans* colonization and molecular mechanisms of azole resistance in autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED) patients. J Antimicrob Chemother 2010; 65: 2505-2513.

[20] Müller FMC, Staudigel A, Salvenmoser S, Tredup A, Miltenberger R, Herrmann JV. Cross-Resistance to medical and agricultural azole drugs in yeasts from the oropharynx of human immunodeficiency virus patients and from environmental Bavarian vine grapes. Antimicrob Agents Chemother 2007; 51: 3014-3016.

[21] Piper P, Mahe Y, Thompson S, Pandjaitan R, Holyoak C, Egner R, Mühlbauer M, Coote P, Kuchler K. The Pdr12 ABC transporter is required for the development of weak organic acid resistance in yeast. The EMBO J 1998; 17: 4257–4265.

[22] Jungwirth H, Kuchler K. Yeast ABC transporters – A tale of sex, stress, drugs and aging. FEBS Lett 2006; 580: 1131–1138.

[23] Bruins MR, Kapil S, Oehme FW. Microbial resistance to metals in the environment. Ecotox Environ Safe 2000; 45: 198-207.

[24] Edelmann A, Krüger M, Schmid J. Genetic relationship between human and animal isolates of *Candida albicans*. J Clin Microbiol 2005; 43: 6164-6166.

Table 1: *Candida* from the gastrointestinal tract of Brazilian psittacines and *in vitro* antifungal susceptibility profile of the recovered isolates

Bird Species	Birds (n)	Collection Site	*Candida* species (a)	Minimum Inhibitory Concentration (µg/mL)			Resistant isolates (n)	
				Amphotericin B	Fluconazole	Itraconazole	Fluconazole	Itraconazole
Aratinga cactorum	22	Oral cavity	*C. famata* (1)	1	8	0.0625	-	-
			C. parapsilosis (4)	0.25 - 1	0.25 – 16	0.03125 – 0.25	1	-
			C. tropicalis (10)	0.125 - 1	0.5 – 250	0.25 - 64	4	2
		Crop	*C. tropicalis* (7)	0.125 – 0.5	0.5 – 32	0.0625 - 8	3	2
		Cloaca	*C. parapsilosis* (2)	0.25 – 0.5	1 – 4	0.0625 – 0.125	-	-
			C. tropicalis (8)	0.125 - 1	1 – 250	0.03125 - 64	6	4
Amazona aestiva	26	Oral cavity	*C. albicans* (2)	0.25	0.5 – 8	0.0625 - 1	1	1
			C. famata (5)	0.25 - 1	0.5 – 4	0.03125 – 0.125	-	-
			C. guilliermondii (3)	0.5 - 1	1 – 4	0.03125 – 0.125	-	-
			C. krusei (4)	2	16	0.03125 – 0.125	-	-
			C. parapsilosis (7)	0.25 - 1	0.25 – 2	0.03125 – 0.25	-	-
			C. tropicalis (11)	0.125 - 1	0.25 – 250	0.03125 - 32	2	2
		Crop	*C. albicans* (1)	0.5	0.25	0.5	-	-
			C. famata (3)	0.125 – 0.5	1 – 4	0.03125 – 0.125	-	-
			C. guilliermondii (1)	1	0.5	0.0625	-	-
			C. krusei (2)	2	16	0.0625 – 0.125	-	-
			C. parapsilosis (3)	1	1 – 4	0.03125 – 0.25	-	-
			C. tropicalis (1)	0.5	0.25	0.0625	-	-
		Cloaca	*C. famata* (1)	0.5	8	0.125	-	-
			C. parapsilosis (6)	0.25 - 1	0.5 – 64	0.03125 – 0.25	1	-
			C. tropicalis (1)	0.0625	2	0.0625	-	-
Amazona amazonica	6	Oral Cavity	*C. parapsilosis* (1)	1	1	0.125	-	-
			C. tropicalis (3)	0.5 - 1	0.25 – 250	0.0625 - 64	2	2
		Crop	*C. krusei* (2)	2	16	0,0625 – 0.125	-	-
			C. parapsilosis (1)	0.5	1	0.0625	-	-
			C. tropicalis (2)	0.25 – 0.5	0.25 – 250	0.0625 - 64	1	1
Ara ararauna	5	Oral Cavity	*C. albicans* (2)	0.5	2 – 8	1 - 8	1	2
		Crop	*C. albicans* (1)	0.0625	250	125	1	1
			C. parapsilosis (1)	0.0625	1	0.03125	-	-
			C. tropicalis (2)	0.125	250	64	2	2
		Cloaca	*C. albicans* (1)	0.5	1	0.03125	-	-
			C. parapsilosis (1)	1	0.5	0.0625	-	-
			C. tropicalis (1)	1	250	32	1	1

a: number of recovered isolates

Table 2: Minimum inhibitory concentration of fluconazole and itraconazole against resistant *Candida* species isolated from animals, before and after the addition of promethazine.

Candida species	n	Minimum inhibitory concentration (µg/mL)		n	Minimum inhibitory concentration (µg/mL)	
		Fluconazole (a)	Fluconazole + Promethazine		Itraconazole	Itraconazole + Promethazine
Candida albicans	19	8 (3)	0,25 (1)	21	1 (3)	0.03125 (2)
		16 (4)	2 (9)		2 (2)	0.0625 (12)
		32 (2)	4 (5)		8 (2)	0.125 (5)
		125 (1)	8 (1)*		16 (1)	0.25 (2)
		250 (9)	16 (3)*		32 (2)	
					64 (10)	
					125 (1)	
Candida parapsilosis	2	64 (1)	1 (1)	-	-	-
		16 (1)	2 (1)	-	-	-
Candida tropicalis	24	8 (3)	1 (2)	19	4 (2)	0.03125 (3)
		16 (2)	2 (1)		8 (3)	0.0625 (10)
		32 (5)	4 (1)		32 (4)	0.125 (4)
		125 (1)	8 (13)*		64 (10)	0.25 (1)
		250 (13)	16 (6)*			0.5 (1)
			32 (1)*			

a: number of isolates; *strains that remained resistant, after the addition of promethazine

7. ASPECTOS METODOLÓGICOS E RESULTADOS OBTIDOS COM LEVAMISOL

7.1 Material e Métodos

7.1.1 Isolados de *Candida* avaliados

Quarenta e oito isolados (12 *C. albicans*, 12 *C. krusei*, 12 *C. parapsilosis* e 12 *C. tropicalis*) de origem veterinária da Micoteca do Centro Especializado em Micologia Médica foram submetidos à análise da sensibilidade ao levamisol. Destes, 37 foram obtidos do trato gastrintestinal de psitacídeos brasileiros, durante o presente estudo, e 11 *C. albicans* foram obtidos do trato gastrintestinal de calopsitas (*Nymphicus hollandicus*) (BRILHANTE et al., 2010). Todos os isolados de *C. albicans* e 9/12 isolados de *C. tropicalis* apresentavam resistência *in vitro* a fluconazol e itraconazol.

7.1.2 Teste de sensibilidade a antifúngicos

O efeito antifúngico do levamisol foi avaliado, por meio da técnica de microdiluição em caldo, como descrito pelo documento M27-A3 do CLSI. Para o ensaio, foi utilizado o cloridrato de levamisol 7,5% (Fort Dodge, Brasil), o qual foi testado no intervalo de concentração de 0,037 a 18,75 mg/mL. Os inóculos das cepas testadas foram preparados a partir de colônias com 24 horas de crescimento em ágar batata dextrose, a 35 ºC. Foram adicionados 5 mL de solução salina estéril, em tubos de vidro estéreis, na qual foi diluída uma pequena amostra da colônia, cuja concentração foi ajustada a 0,5 na escala de McFarland (CLSI, 2008; SIDRIM et al. 2010). Posteriormente, os inóculos foram diluídos na razão de 1:100 e, em seguida, de 1:20, em meio RPMI acrescido de L-glutamina (HiMedia, Mumbai, India), tamponado a pH 7 com ácido morfolinopropanossulfônico (MOPS) a 0,165M. A concentração final dos inóculos foi de 0,5 a 2,5 x 10^3 células/mL (CLSI, 2002; SIDRIM et al. 2010). As placas foram incubadas a 35 ºC, por 48 horas.

Para cada isolado testado, foram incluídos poços sem droga, como controle de crescimento, e poços sem inóculo, como controle de esterilidade e todas as cepas foram testadas em duplicata. Como controle de qualidade, para cada ensaio realizado, a cepa *C. krusei* ATCC 6258 foi incluída (CLSI, 2008), a qual foi testada ante ao levamisol e à anfotericina B. A concentração inibitória mínima (CIM) para o levamisol foi determinada como a menor concentração capaz de inibir 80% do crescimento, quando comparado ao poço controle. Adicionalmente, a concentração fungicida mínima (CFM) de levamisol também foi determinada para todas as cepas avaliadas. Para tanto, após a leitura das placas de microdiluição e determinação da CIM, alíquotas de 20 µL foram retiradas dos poços

referentes à CIM e a três concentrações crescentes subsequentes (2xCIM, 4xCIM e 8xCIM) e semeadas em placas contendo ágar batata dextrose. As placas foram incubadas a 35 ºC, durante 48 horas, e foram lidas. A CFM foi definida como a menor concentração na qual não se observou crescimento fúngico no subcultivo (TAWARA et al., 2000).

7.1.3 Efeito do levamisol sobre o biofilme de *Candida* spp.

O efeito do levamisol sobre o biofilme maduro de *Candida* spp. e sobre a formação de biofilme por esses microrganismos também foi avaliado. Para tanto, a formação de biofilme foi induzida em 12 isolados resistentes a derivados azólicos (6 *C. albicans* e 6 *C. tropicalis*) e nas cepas controle *C. parapsilosis* ATCC 22019 e *C. krusei* ATCC 6258.

Inicialmente, esses isolados foram crescidos em ágar Sabouraud dextrose, a 30 ºC, durante 48 horas. Em seguida, os isolados foram crescidos em caldo Sabouraud dextrose, durante 24 horas, a 30 ºC, sob agitação (150 rpm). Após esse período, os tubos com caldo Sabouraud dextrose, contendo o crescimento fúngico, foram centrifugados a 3000 rpm, durante 10 minutos, e o sobrenadante foi descartado. Posteriormente, o *pellet* fúngico foi ressuspenso em meio RPMI, alcançando uma turbidez de 0,5 a 1 na escala McFarland. Os inóculos fúngicos (100 µL) foram adicionados aos poços de uma placa estéril de poliestireno de 96 poços de fundo chato. As placas foram então incubadas a 37 ºC, durante 48 horas (PEETERS et al., 2008).

Com o intuito de avaliar o efeito de drogas sobre a formação de biofilme, no momento da inoculação das placas, o levamisol foi adicionado em três concentrações distintas: CIM, 2xCIM e 4xCIM ante a cada isolado avaliado e ante às cepas controle. Portanto, para cada isolado testado, oito poços foram utilizados: 1) formação de biofilme na ausência de levamisol (controle); 2) formação de biofilme após a adição de levamisol na concentração CIM; 3) formação de biofilme após a adição de levamisol na concentração 2xCIM; 4) formação de biofilme após a adição de levamisol na concentração 4xCIM, sendo todos os tratamentos avaliados em duplicata. Para as cepas controle, a inibição da formação de biofilme por meio da adição de anfotericina B (droga controle) nas concentrações 10xCIM, 20xCIM e 50xCIM também foi avaliada. Em cada placa, foram incluídos controles positivo e negativo e controle da droga.

Após o período de incubação, o sobrenadante de todos os poços avaliados foi cuidadosamente removido e as células aderidas foram lavadas três vezes com solução PBS-Tween. Em seguida, todos os poços foram lavados com metanol 100% (100 µL), o sobrenadante foi removido e as placas foram deixadas a temperatura ambiente até secagem total dos poços. Após essa etapa, 100 µL de solução de cristal violeta a 0,3% foram

adicionados a cada poço, onde o corante foi mantido por 20 minutos. Em seguida, o cristal violeta foi removido e os poços foram lavados duas vezes com água destilada estéril. Finalmente, 150 μL de ácido acético a 33% foram adicionados a cada poço corado e, após 30 segundos, os volumes foram retirados. As placas foram imediatamente lidas por espectrofotometria em leitora própria para placas de fundo chato, a 590 nm (PEETERS et al., 2008) e os resultados da densidade óptica foram analisados.

Com o intuito de avaliar o efeito do levamisol sobre o biofilme formado, após 48 horas de incubação das placas inoculadas, o levamisol foi adicionado em três concentrações diferentes: CIM, 10xCIM e 20xCIM, contra cada isolado avaliado e as cepas controle. Assim, para cada isolado de *Candida* testado, oito poços foram utilizados: 1) manutenção do biofilme na ausência de levamisol (controle); 2) manutenção do biofilme após a adição de levamisol na concentração CIM; 3) manutenção do biofilme após a adição de levamisol na concentração 10xCIM; 4) manutenção do biofilme após a adição de levamisol na concentração 20xCIM, sendo todos os tratamentos avaliados em duplicata. Para as cepas controle, a inibição do biofilme formado, por meio da adição de anfotericina B (droga controle) nas concentrações 10xCIM, 20xCIM e 50xCIM, também foi avaliada. Em cada placa, foram incluídos controles positivo e negativo e controle da droga. Após a adição da droga a ser testada, as placas foram incubadas por mais 48 horas, a 37 ºC, e os procedimentos restantes foram realizados como descrito previamente.

7.1.4 Análise Estatística

Para comparar as CIMs e CFMs do levamisol entre as diferentes espécies de *Candida*, foi aplicada a Análise de Variância, com nível de significância de 5%. O efeito do levamisol sobre a formação de biofilme e sobre a manutenção do biofilme formado também foi avaliado, por meio do teste T de Student para amostras pareadas, com um nível de significância de 5%.

7.2 Resultados

Os valores de CIM variaram de 0,58 a 2,34 mg/mL, com médias geométricas bastante similares entre as espécies testadas: 1,27 mg/mL para *C. albicans*, 1,17 mg/mL para *C. krusei* e *C. parapsilosis* e 1,31 mg/mL para *C. tropicalis.* Os valores de CFM obtidos variaram de 2,34 a 9,37 mg/mL, apresentando médias geométricas mais divergentes: 9,37 mg/mL para *C. albicans*, 3,94 mg/mL para *C. krusei* e *C. parapsilosis* e 5,57 mg/mL para *C. tropicalis* (Tabela 5).

Observou-se que o levamisol inibiu a formação de biofilme nas concentrações 2xCIM e 4xCIM, mas não na CIM, e que essa inibição seguiu um padrão dose-dependente,

como demonstrado pela redução significativa na densidade óptica entre os tratamentos controle e 2xCIM (P=0,0139), CIM e 2xCIM (P=0,0000) e 2xCIM e 4xCIM (P=0,0000). Adicionalmente, o levamisol também apresentou atividade inibitória contra o biofilme maduro, em todas as considerações testadas, seguindo um padrão dose-dependente, como demonstrado pela redução significativa na densidade óptica entre os tratamentos controle e CIM (P=0,0184), CIM e 10xCIM (P=0,0007) e 10xCIM e 20xCIM (P=0.0000).

Tabela 1: Médias geométricas e distribuição das concentrações inibitórias mínimas CIMs e das concentrações fungicidas mínimas (CFMs) de levamisol contra *Candida* spp. isoladas de aves

***Candida* spp.**	**Levamisol**		**Anfotericina B**
	CIM (mg/mL)	CFM (mg/mL)	
***C. albicans* (12)**	1,17 (11)	9,37 (15)	0,25 – 1
	2,34 (1)		
Média geométrica	1,27	9,37	-
***C. krusei* (12)**	1,17 (12)	2,34 (5)	2
		4,68 (5)	
		9,37 (2)	
Média geométrica	1,17	3,94	-
***C. parapsilosis* (12)**	1,17 (12)	2,34 (3)	0,0625 – 1
		4,68 (9)	
Média geométrica	1,17	3,94	-
***C. tropicalis* (12)**	0,58 (1)	4,68 (9)	0,125 – 1
	1,17 (10)	9,37 (3)	
	2,34 (2)		
Média geométrica	1,31	5,57	-
***C. krusei* ATCC**	1,17	2,34	1

8. CONCLUSÃO

1. As leveduras do gênero *Candida* fazem parte da microbiota do trato gastrintestinal, da pele e do sistema geniturinário de animais. Nas aves, esses microrganismos são prontamente isolados do trato gastrintestinal, com destaque para as espécies *C. tropicalis*, *C. parapsilosis*, *C. albicans* e *C. famata*, as quais apresentaram um maior número de isolados. Ao comparar as diferentes espécies de aves avaliadas, foi possível observar que as taxas de isolamento são variáveis entre as espécies animais, no entanto, as espécies de *Candida* isoladas não apresentaram grande variação. Com base no exposto, torna-se evidente a ampla distribuição dessas leveduras no reino animal, as quais, no geral, estão em equilíbrio com o hospedeiro, não ocasionando manifestações clínicas.

2. Dos 126 isolados avaliados, 33 (26.2%) apresentaram resistência primária a fluconazol e itraconazol. Essa taxa de resistência é relativamente elevada, quando comparada àquelas de estudos com cepas de origem humana. As espécies *C. albicans* e *C. tropicalis* foram as que apresentaram um maior número de isolados resistentes.

3. A inibição da atividade das bombas de efluxo Cdr1 e Cdr2 reverteu o fenótipo de resistência de todos os isolados resistentes a itraconazol. No entanto, para fluconazol, somente 46,7% das cepas resistentes tornaram-se sensíveis. Com base no exposto, observou-se que a resistência a itraconazol nos isolados avaliados está exclusivamente associada ao aumento da atividade das bombas Cdr1 e Cdr2, enquanto que a resistência ao fluconazol, na maioria dos isolados, foi multifatorial, envolvendo não só a atividade dessas bombas, como outros mecanismos ainda não elucidados.

4. O imidazol levamisol apresentou atividade inibitória contra *Candida* spp., tanto na fase planctônica, quanto na forma de biofilme. Essa droga não só inibiu a formação de biofilme, como agiu sobre o biofilme formado. Esses dados enaltecem a importância da busca por novos compostos com atividade antifúngica e abrem perspectivas para a realização de pesquisas com o intuito de compreender o mecanismo da ação antifúngica do levamisol e de validar o uso dessa droga *in vivo*, no tratamento de candidíases.

9. REFERÊNCIAS BIBLIOGRÁFICAS

ABRÃO, F. O.; FREITAS, C. E. S.; DUARTE, E. R.; GERASEEV, L. C.; BARRETO, S. M. P.; MEDEIROS, A. O.; ROSA, C. A. Leveduras no rúmen de caprinos e bovinos de corte criados em pastagem tropicais. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, v. 63, p. 526-529, 2011.

BARRETT-BEE, K.; HAYES, Y. WILSON, R. G.; RYLEY, J. F. A comparison of phospholipase activity, cellular adherence and pathogenicity of yeasts. *Journal of General Microbiology*, v. 131, p. 1217-1221, 1985.

BRILHANTE, R. S.; CASTELO-BRANCO, D. S. C. M.; SOARES, G. D.; ASTETE-MEDRANO, D. J.; MONTEIRO, A. J.; CORDEIRO, R. A.; SIDRIM, J. J.; ROCHA, M. F. Characterization of the gastrointestinal yeast microbiota of cockatiels (*Nymphicus hollandicus*): a potential hazard to human health. *Journal of Medical Microbiology*, v. 59**,** p. 718-723, 2010.

BRILHANTE, R. S.; PAIVA, M. A. N.; SAMPAIO, C. M. S.; TEIXEIRA, C. E. C.; CASTELO-BRANCO, D. S. C. M.; LEITE, J. J. G.; MOREIRA, C. A.; SILVA, L. P.; CORDEIRO, R. A.; MONTEIRO, A. J.; SIDRIM, J. J. C.; ROCHA, M. F. G. Yeasts from *Macrobrachium amazonicum*: a focus on antifungal susceptibility and virulence factors of *Candida* spp. FEMS *Microbiology Ecolology*, v. 76, p. 268 – 277, 2011.

BRITO, E. H. S.; FONTENELLE, R. O. S.; BRILHANTE, R. S. N.; CORDEIRO, R. A.; MONTEIRO, A. J.; SIDRIM, J. J. C.; ROCHA, M. F. G. The anatomical distribution and antimicrobial susceptibility of yeast species isolated from healthy dogs. *The Veterinary Journal*, v. 182, p. 320-326, 2009a.

BRITO, E. H. S.; FONTENELLE, R. O. S.; BRILHANTE, R. S. N.; CORDEIRO, R. A.; SIDRIM, J. J. C.; ROCHA, M. F. G. Candidose na medicina veterinária: um enfoque micológico, clínico e terapêutico. *Ciência Rural*, v.39, p.2655-2664, 2009b.

BRITO, E. H. S.; FONTENELLE, R. O. S.; BRILHANTE, R. S. N.; CORDEIRO, R. A.; SOARES- JUNIOR F. A.; MONTEIRO, A. J.; SIDRIM, J. J. C.; ROCHA, M. F. G. Phenotypic characterization and in vitro antifungal sensitivity of *Candida* spp. and *Malassezia pachydermatis* strains from dogs. *The Veterinary Journal*, v.174, p.147–153, 2007.

BROWN, R. M.; THOMPSON, C. A.; MOHAMED, F. M. Systemic candidiasis in an apparently immunocompetent dog. *Journal of Veterinary Diagnosis Investigation*, v.17, p. 272–276, 2005.

BRUINS, M. R.; KAPIL, S.; OEHME, F. W. Microbial resistance to metals in the environment. *Ecotoxicology and Environmental Safety*, v. 45, p. 198-207, 2000.

CAFARCHIA, C.; CAMARDA, A.; ROMITO, D.; CAMPOLO, M.; QUAGLIA, N. C.; TULLIO, D.; OTRANTO, D. Occurrence of yeasts in cloacae of migratory birds. *Mycopathologia*, v. 161, p. 229-234, 2006a.

CAFARCHIA, C.; ROMITO, C.; COCCIOLI, C.; CAMARDA, A.; OTRANTO, D. Phospholipase activity of yeasts from wild birds and possible implications for human diseases. *Medical Mycology*, v. 46, p. 1-6, 2008.

CAFARCHIA, C.; ROMITO, D.; IATTA, R.; CAMARDA, A.; MONTAGNA, M. T.; OTRANTO, D. Role of birds of prey as carriers and spreaders of *Cryptococcus neoformans* and other zoonotic yeasts. *Medical Mycology*, v. 44, p. 485-492, 2006b.

CANNON, R. D.; LAMPING, E.; HOLMES, A. R.; NIIMI, K.; BARET, P. V.; KENIYA, M. V.; TANABE, K.; NIIMI, M.; GOFFEAU, A.; MONK, B. C. Efflux-mediated antifungal drug resistance. *Clinical Microbiology Reviews*, v. 22, p.291-321, 2009.

CARREGARO, F. B.; SPANAMBERG, A.; SANCHES, E. M. C.; ARGENTA, J. S.; PEREIRA, D. I. B.; ZANETTE, R.; SANTURIO, J. M.; BARCELLOS, D. E. S. N.; FERREIRO, L. Fungal microbiota isolated from healthy pig skin. *Acta Scientiae Veterinariae*, v. 38, p.147-153, 2010.

CARVALHO, L. R.; FARIAS, L. M.; NICOLI, J. R.; SILVA, M. C. F.; CORSINO, A. T. S. M.; LIMA, L. A.; REDONDO, R. A. F.; FERREIRA, P. C. P.; PINTO, M. E. B. M. Dominant culturable bacterial microbiota in the digestive tract of the American black vulture (*Coragyps atratus* Bechstein 1793) and search for antagonistic substances. *Brazilian Journal of Microbiology*, v.34, p. 218-224, 2003.

CHAN, Y. Y.; ONG, Y. M.; CHUA, K. L. Synergistic interaction between phenothiazines and antimicrobial agents against *Burkholderia pseudomallei*. *Antimicrobial Agents and Chemotherapy*, v. 51, p. 623-630, 2007.

CLEFF, M. B.; LIMA, A. P.; FARIA, R. O.; MEINERZ, A. R. M.; ANTUNES, T. A.; ARAÚJO, F. B.; NASCENTE, P. S.; NOBRE, M. O.; MEIRELES, M. C. A. Isolation of *Candida* spp. from vaginal microbiota of healthy canine females during estrous cycle. *Brazilian Journal of Microbiology*, v. 36, p. 201-204, 2005.

CLEFF, M. B.; SILVA, G. M.; MEINERZ, A. R. M.; MADRID, I. M.; MARTINS, A. A.; FONSECA, A. O.; NASCENTE, P. S.; MEIRELES, M. C. A.; MELLO, J. R. B.. Infecção cutânea em cão por *Candida albicans. Veterinária e Zootecnia*, v.14, p. 164-168, 2007.

CLSI, 2008. Clinical and Laboratory Standards Institute. Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard M27-A3.

COSTA, K. R. C.; FERREIRA, J. C.; KOMESU, M. C.; CANDIDO, R. C. *Candida albicans* and *Candida tropicalis* in oral candidosis: quantitative analysis, exoenzyme activity and antifungal drug sensitivity. *Mycopathologia*, v. 167, p. 73-79, 2009.

DE HOOG, G. S.; GUARRO, J.; GENÉ, J.; FIGUEIRAS, M. J. *Atlas of Clinical Fungi*. The Nederlands: Centraalbureau voor Schimmslcultures, 2ª ed. Baarn, 2000. p. 130–143, 156–160, 164–174.

DIEZMANN, S.; COX, C. J.; SCHÖNIAN, G.; VILGALYS, R. J.; MITCHELL, T. G. Phylogeny and Evolution of Medical Species of Candida and Related Taxa: a Multigenic Analysis. *Journal of Clinical Microbiology*, v. 42, p. 5624–5635, 2004.

EDELMANN, A.; KRÜGER, M.; SCHMID, J. Genetic Relationship between human and animal isolates of *Candida albicans*. *Journal Clinical Microbiology*, v. 43, p. 6164-6166, 2005.

FENG, L.; WAN, Z.; WANG, X.; LI, R.; LIU, W. Relationship between antifungal resistance of fluconazole resistant *Candida albicans* and mutations in *ERG11* gene. *Chinese Medical Journal*, v.123, p.544–548, 2010.

FOTEDAR, R.; AL-HEDAITHY, S. S. A. Comparison of phospholipase and proteinase activity in *Candida albicans* and *C. dubliniensis*. *Mycoses*, v. 48, p. 62-67, 2005.

GALGÓCZY, L.; BÁCSI, A.; HOMA, M.; VIRÁGH, M.; PAPP, T.; VÁGVÖLGYI, C. *In vitro* antifungal activity of phenothiazines and their combination with amphotericin B against different *Candida* species. *Mycoses*, v. 54, p. e737-e743, 2011

GARCIA, M. E.; LANZAROT, V. L.; RODAS, V. L.; COSTAS, E.; BLANCO, J. L. Fungal flora in the trachea of birds from a wildlife rehabilitation centre in Spain. *Veterinarni Medicina*, v. 52, p. 464-470, 2007.

GARCÍA-MARTOS, P.; GARCÍA-AGUDO, L.; RUIZ-ARAGÓN; SALDARREAGA, A.; MARÍN, P. Asimilación de carbohidratos por cepas de *Rhodotorula glutinis* de origen clínico y ambiental. *Revista Iberoamericana de Micología,* v. 21, p. 90-92, 2004.

GATESOUPE, F. J. Live yeasts in the gut: Natural occurrence, dietary introduction, and their effects on fish health and development. *Aquaculture*, v. 267, p. 20–30, 2007.

GERSHENSON, R. T.; MELIDONE, R.; SUTHERLAND-SMITH, J.; ROGERS, C. L. Abdominal aortic aneurysm associated with systemic fungal infection in a German Shepherd dog. *Journal of the American Animal Hospital Association*, v. 47, p. 45-49, 2011.

GELLIS, S. Evaluating and treating the gastrointestinal system. In: HARRISON, G. J.; LIGHTFOOT, T. L. *Clinical Avian Medicine*. Palm Beach, Flórida: Spix Publishing, 2006. cap.14, p. 411–440.

GHANNOUM, M. A. Potential role of phospholipases in virulence and fungal pathogenesis. *Clinical Microbiology Reviews,* v. 13, p.122-143, 2000.

GUÉHO, E.; IMPROVISI, L.; DE HOOG, G. S.; DUPONT, B. *Trichosporon* on humans: a practical account. *Mycoses*, v. 37, p. 3-10, 1993.

GUENTHER, S.; GROBBEL, M.; LÜBKE-BECKER, A.; FRIEDRICH, A. G. N. D.; WIELER, L. H.; Ewers, C. Antimicrobial resistance profiles of *Escherichia coli* from common European wild bird species. *Veterinary Microbiology*, v.144, p.219–225, 2010.

HARRIOTT, M. M.; NOVERR, M. C. Importance of *Candida*–bacterial polymicrobial biofilms in disease. *Trends Microbiology*, v.19, p.557-563, 2011.

HERNANDEZ-DIVERS, S. J. Pulmonary candidiasis caused by *Candida albicans* in a greek tortoise (*Testudo graeca*) and treatment with intrapulmonary amphotericin B. *Journal of Zoo and Wildlife Medicine*, v.32, p.352–359, 2001.

HESELTINE, J. C.; PANCIERA, D. L.; SAUNDERS, G. K. Systemic candidiasis in a dog. *Journal of the American Veterinary Medical Association,* v.223, p.821-824, 2003.

HOSPENTHAL, D. R.; MURRAY, C. K.; RINALDI, M. G. The role of antifungal susceptibility testing in the therapy of candidiasis. *Diagnostic Microbiology and Infectious Disease*, v. 48, p.153-160, 2004.

IBRAHIM, A. S.; MIRBOD, F.; FILLER, S. G.; BANNO, Y.; COLE, G. T.; KITAJIMA, Y.; EDWARDS JR., J. E.; NOZAWA, Y.; GHANNOUM, M. A. Evidence implicating phospholipase as a virulence factor of *Candida albicans*. *Infection and Immunity*, v. 63, p. 1993-1998, 1995.

INSTITUTO CHICO MENDES DE CONSERVAÇÃO DA BIODIVERSIDADE (2008) Plano de ação nacional para a conservação de aves de rapinas. ICMBio, Brasília.

INTEGRATED TAXONOMIC INFORMATION SYSTEM (ITIS). www.itis.gov (Accessado em 21 de Junho 2011)

JACOBSON, E. R.; CHEATWOOD, J. L.; MAXWELL, L. K. Mycotic Diseases of Reptiles. *Seminars in Avian and Exotic Pet Medicine*, v. 9, p. 94-101, 2000.

JIN, Y.; LIN, D. Fungal urinary tract infections in the dog and cat: a retrospective study (2001-2004). *Journal of the American Animal Hospital Association*, v.41, p.373-381, 2005.

JOHNSON, E. M. Issues in antifungal susceptibility testing. *Journal of Antimicrobial Chemotherapy*, v. 61, p. il3-il8, 2008.

JOHNSON, S. K.; BUENO, S. L. S. Health Management. In: NEW, M. B.; VALENTI, W.C. (Eds.), Freshwater prawn culture: the farming of *Macrobrachium rosenbergii*, 443p. London: Blackwell Science, p. 18-40, 2000.

JUNGWIRTH, H.; KUCHLER, K. Yeast ABC transporters – A tale of sex, stress, drugs and aging. *FEBS Letter* v. 580, p. 1131–1138, 2006.

KANAFANI, Z. A.; PERFECT, J. R. Resistance to antifungal agents: mechanisms and clinical impact. *Clinical Infectious Diseases*, v. 46, p. 120-128, 2008.

KANO, R.; SAKAMOTO, Y.; HANAHACHI, A.; KAMATA, H.; FUKUDA, Y.; FUJIWARA, K.; HASEGAWA, A. Molecular identification of *Candida parapsilosis* from crop mucosa in a cockatiel. *Journal of Veterinary Diagnostic Investigation*, v. 13, p. 437-439, 2001.

KECK, N.; LIBERT, C.; RISPAIL, P.; ALBARIC, O. Systemic candidosis in a guanaco (*Lama guanicoe*). *Veterinary Record*, v.164, p. 245-246, 2009.

KIVARIA, F. M.; NOORDHUIZEN, J. P. T. M. A retrospective study of the aetiology and temporal distribution of bovine clinical mastitis in smallholder dairy herds in the Dar es Salaam region of Tanzania. *Veterinary Journal*, v.173, p.617-622, 2007.

KLAPHAKE, E.; CLANCY, J. Raptor gastroenterology. *Veterinary Clinics Exotic Animal Practice*, v. 8, p. 307-327, 2005.

KOBAYASHI, C. Distribution of yeast-form fungi in oral cavity of dog. *International Journal of Oral Medicine Science*, v. 7, p. 40-44, 2008.

KOFLA, G.; TURNER, V.; SCHULZ, B.; STORCH, U.; FROELICH, D.; ROGNON, B.; COSTE, A. T.; SANGLARD, D.; RUHNKE, M. Doxorubicin induces drug efflux pumps in *Candida albicans*. *Medical Mycology*, v. 49, p. 132–142, 2011.

KOLACZKOWSKI, M.; MICHALAK, K.; MOTOHASHI, N. Phenothiazines as potent modulators of yeast multidrug resistance. *International Journal of Antimicrobial Agents*, v. 22, p. 279-283, 2003.

KRIENGKAUYKIAT, J.; PORTER, E.; LOMOVSKAYA, O.; WONG-BERINGER, A. Use of an efflux pump inhibitor to determine the prevalence of efflux pump-mediated fluoroquinolone resistance and multidrug resistance in *Pseudomonas aeruginosa*. *Antimicrobial Agents Chemotherapy*, v. 49, p. 565–570, 2005.

LAGROU, K.; VAN ELDERE, J.; KEULEERS, S.; HAGEN, F.; MERCKX, R.; VERHAEGEN, J.; PEETERMANS, W. E.; BOEKHOUT, T. Zoonotic transmission of *Cryptococcus neoformans* from a magpie to an immunocompetent patient. *Journal of Internal Medicine*, v. 257, p. 385-388, 2005.

LAI, W. H.; LU, S. Y.; ENG, H. L. Levamisole aids in treatment of refractory oral candidiasis in two patients with thymoma associated with myasthenia gravis: report of two cases. *Chang Gung Medical Journal*, v. 25, p. 606-611, 2002.

LEAÑO, E. M.; LIO-PO, G. D.; NADONG, L. A.; TIRADO, A. C.; SADABA, R. B.; GUANZON-JR., N. G. Mycoflora of the 'green water' culture system of tiger shrimp Penaeus monodon Fabricius, *Aquaculture Research*, v. 36, p. 1581-1587, 2005.

LEE, H. A.; HONG, S.; CHOE, O.; KIM, O. Mural folliculitis and alopecia with cutaneous candidiasis in a beagle dog. *Laboratory Animal Research*, v. 27, p. 63-65, 2011.

LEELAYUWAT, C.; ROMPHRUK, A.; LULITANOND, A.; TRAKULSOMBOON, S.; THAMLIKITKUL, V. Genotype analysis of *Burkholderia pseudomallei* using randomly amplified polymorphic DNA (RAPD): indicative of genetic differences amongst environmental and clinical isolates. *Acta Tropica*, v. 77, p. 229–237, 2000.

LU, C. C.; TANG, K. F. J.; CHEN, S. N. Identification and genetic characterization of yeasts isolated from freshwater prawns, *Macrobrachium rosenbergii* de Man, in Taiwan. *Journal of Fish Diseases*, v. 21, p. 185–192, 1998.

MANASTIR, L.; ERGON, M. C.; YÜCESOY, M. Investigation of mutations in *ERG11* gene of fluconazole resistant *Candida albicans* isolates from Turkish hospitals. *Mycoses*, v. 54, p. 99-104, 2009.

MANCIANTI, F.; NARDONI, S.; CECCHERELLI, R. Occurrence of yeasts in psittacine droppings from captive birds in Italy. *Mycopathologia*, v. 153, p. 121-124, 2001.

MARROW, J.; WHITTINGTON, J. K.; MITCHELL, M.; HOYER, L. L.; MADDOX, C. Prevalence and antibiotic-resistance characteristics of *Enterococcus* spp. isolated from free-living and captive raptors in central Illinois. *Journal of Wildlife Disease*, v.45, p. 302–313, 2009.

MARTIN, R. J.; ROBERTSON, A. P. Mode of action of levamisole and pyrantel, antihelmintic resistance, E153 and Q57. *Parasitology*, v. 134, p. 1093-1104, 2007.

MARTINS, M.; DASTIDAR, S. G.; FANNING, S.; KRISTIANSEN, J. E.; MOLNAR, J.; PAGES, J. M.; SCHELZ, Z.; SPENGLER, G.; VIVEIROS, M.; AMARAL, L. Potential role of non-antibiotics (helper compounds) in the treatment of multidrug-resistant Gram-negative infections: mechanisms for their direct and indirect activities. *International Journal Antimicrobial Agents*, v. 31, p. 198–208, 2008.

MELVILLE, P. A.; COGLIATI, B.; MANGIATERRA, M. B. B. C. D.; PERES, M. R.; MOURA, S. C. A.; MATSUDA, L.; KIM, A.; BENITES, N. R. Determinação da microbiota presente na cloaca e orofaringe de avestruzes (*Struthio camelus*) clinicamente sadios. *Ciência Rural*, v.34, p.1871-1876, 2004.

MEYER, M. H.; LETSCHER-BRU, V.; WALLER, J.; LUTZ, P.; MARCELLIN, L.; HERBRECHT, R. Chronic disseminated *Trichosporon asahii* infection in a leukemic child. *Clinical Infectious Diseases*, v. 35, p. e22-e25, 2002.

MILAN, E. P.; ZAROR, L. Leveduras: identificação laboratorial. In: SIDRIM, J. J. C.; ROCHA, M. F. G. *Micologia médica à luz de autores contemporâneos*. Rio de Janeiro: Guanabara Koogan, 2004. cap. 6, p. 50-62.

MORETTI, A.; FIORETTI, D. P.; BONCIO, L.; PASQUALI, P.; DEL ROSSI, E. Isolation of *Candida rugosa* from turkeys. *Journal of Veterinary Medicine*, v. 47, p. 433-439, 2000.

MORETTI, A.; POSTERARO, B.; BONCIO, L.; MECHELLI, L.; DE GASPERIS, E.; AGNETTI, F.; RASPA, M. Diffuse cutaneous candidiasis in a dog. Diagnosis by PCR-REA. *Revista Iberoamericana de Micologia*, v.21, p.139-142, 2004.

MORRIS, P. J.; JOHNSON, W. R.; PISANI, J.; BOSSART, G. D.; ADAMS, J.; REIF, J. S.; FAIR, P. A. Isolation of culturable microorganisms from free-ranging bottlenose dolphins (*Tursiops truncatus*) from the southeastern United States. *Veterinary Microbiology*, v. 148, 440-447, 2011.

MORTENSEN, K. L.; MELLADO, E.; LASS-FLÖRL, C.; RODRIGUEZ-TUDELA, J. L.; JOHANSEN, H. K.; ARENDRUP, M. C. Environmental study of azole-resistant *Aspergillus fumigatus* and other aspergilli in Austria, Denmark, and Spain. *Antimicrobial Agents and Chemotherapy*, v. 54, p.4545–4549, 2010.

MÜLLER, F. M. C.; STAUDIGEL, A.; SALVENMOSER, S.; TREDUP, A.; MILTENBERGER, R.; HERRMANN, J. V. Cross-Resistance to medical and agricultural azole drugs in yeasts from the oropharynx of human immunodeficiency virus patients and from environmental Bavarian vine grapes. *Antimicrobrial Agents and Chemotherapy*, v. 51, p. 3014-3016, 2007.

MUNIZ, M. M.; TAVARES, M. S. P.; MEYER, W.; NOSANCHUK, J. D.; ZANCOPÉ-OLIVEIRA, R. M. Comparison of different DNA-based methods for molecular typing of *Histoplasma capsulatum*. *Applied and Environmental Microbiology*, v. 76, p. 4438-4447, 2010.

NARDONI, S.; PAPINI, R.; MARCUCCI, G. M.; MANCIANTI, F. Survey on the fungal flora of the cloaca of healthy pet reptiles. *The Révue de Médecine Vétérinaire*, v. 159, p. 159-165, 2008.

NAWANGE, S. R.; SINGH, K.; NAIDU, J.; SINGH, S. M. Naturally acquired systemic dual infection caused by *Candida famata* (*Debaryomyces hansenii*) and *Candida catenulata* in albino rats bred for sale in the market at Jabalpur (Madhya Pradesh), India. *Mycoses*, v. 53, p. 173–175, 2009.

OMBRELLA, A. M.; RACCA, L.; RAMOS, L. Actividades proteinasa y fosfolipasa de aislamientos de *Candida albicans* provenientes de secreciones vaginales con distintos valores de pH. *Revista Iberoamericana de Micología*, v. 25, p. 12-16, 2008.

OSORIO, C.; FLETCHER, O.; DYKSTRA, M. J.; POST, K.; BARNES, H. J. Comb candidiasis affecting roosters in a broiler breeder flock. *Avian Diseases*, v. 51, p. 618-622, 2007.

PALACIO, A.; VILLAR, J.; ALHAMBRA, A. Epidemiología de las candidiasis invasoras em población pediátrica y adulta. *Revista Iberoamericana de Micología*, v. 26, p. 2-7, 2009.

PAWAIYA, R. V. S.; SHARMA, A. K.; SWARUP, D.; SOMVANSHI, R. Pathology of mycotic gastritis in a wild Indian freshwater/marsh crocodile (Mugger; *Crocodylus palustris*): a case report. *Veterinarni Medicina*, v. 56, p. 135-139, 2011.

PEETERS, E.; NELIS, H. J.; COENYE, T. Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. *Journal of Microbiological Methods*, v. 72, p.157–165, 2008.

PEREA, S.; LOPEZ-RIBOT, J. L.; KIRKPATRICK, W. R.; MCATEE, R. K.; SANTILLAN, R. A.; MARTINEZ, M.; CALABRESE, D.; SANGLARD, D.; PATTERSON, T. F. Prevalence of molecular mechanisms of resistance to azole antifungal agents in *Candida albicans* strains displaying high-level fluconazole resistance isolated from human immunodeficiency virus-infected patients. *Antimicrobial Agents and Chemotherapy*, v. 45, p. 2676–2684, 2001.

PFALLER, M. A., ANDES, D., DIEKEMA, D. J., ESPINEL-INGROFF, A., SHEEHAN, D. Wild-type MIC distributions, epidemiological cutoff values and species-specific clinical

breakpoints for fluconazole and *Candida*: Time for harmonization of CLSI and EUCAST broth microdilution methods. *Drug Resistance Updates*, v. 13, p. 180-195, 2010.

PFALLER, M. A; DIEKEMA, D. J. Epidemiology of invasive candidiasis, a persistent public health problem. *Clinical Microbiology Reviews*, v. 20, p. 133-163, 2007.

PFALLER, M. A.; ANDES, D.; DIEKEMA, D. J.; ESPINEL-INGROFF, A.; SHEEHAN, D. Wild-type MIC distributions, epidemiological cutoff values and species-specific clinical breakpoints for fluconazole and *Candida*: Time for harmonization of CLSI and EUCAST broth microdilution methods. *Drug Resist Update*, v. 13, p. 180-195, 2010a.

PFALLER, M. A.; DIEKEMA, D. J.; GIBBS, D. L.; NEWELL, V. A.; ELLIS, D.; TULLIO, V.; RODLOFF, A.; FU, W.; LING, T. A. The Global Antifungal Surveillance Group. Results from the ARTEMIS DISK Global Antifungal Surveillance Study, 1997 to 2007: a 10.5-Year analysis of susceptibilities of *Candida* Species to fluconazole and voriconazole as determined by CLSI Standardized Disk Diffusion. *Journal of Clinical Microbiology,* v. 48: p. 1366–1377, 2010.

PIPER, P.; MAHE, Y.; THOMPSON, S.; PANDJAITAN, R.; HOLYOAK, C.; EGNER, R.; MÜHLBAUER, M.; COOTE, P.; KUCHLER, K. The Pdr12 ABC transporter is required for the development of weak organic acid resistance in yeast. *The EMBO Journal*, v. 17, p. 4257–4265, 1998.

PRICE, M. F.; WILKINSON, I. D.; GENTRY, L. O. Plate method for detection of phospholipase activity of *Candida albicans*. *Sabouraudia* v. 22, p. 201–207, 1982.

QUINDÓS, G.; ALONSO-VARGAS, R.; HELOU, S.; ARECHAVALA, A.; MARTÍN-MAZUELOS, E.; NEGRONI, R. Evaluación micológica de uno nuevo medio de cultivo cromógeno (Candida ID®) para el aislamiento e identificación presuntiva de *Candida albicans* y otras levaduras e interés médico. *Revista Iberoamericana de Micología*, v. 18, p. 23-28, 2001.

REX, J. H.; PFALLER, M. A. Has antifungal susceptibility testing come of age? *Clinical Infectious Diseases*, v. 35, p. 982-989, 2002.

ROBERTS, M.; BRAND, S.; MALINIAK, E. The biology of captive prehensile-tailed porcupines, *Coendou prehensilis*. *Journal Mammalogy*, v. 66, p. 476-482, 1985.

ROCHA, M. F. G.; SIDRIM, J. J. C. Drogas Antifúngicas Utilizadas na Terapêutica Contemporânea. In: SIDRIM, J. J. C.; ROCHA, M. F. G. *Micologia médica à luz de autores contemporâneos*. Rio de Janeiro: Guanabara Koogan, 2004. cap. 6, p. 50-62.

ROSTAMI, A.; SHIRANI, D.; SHOKRI, H.; KHOSRAVI, A.R.; DAIEGHAZVINI, R.; TOOTIAN, Z. Fungal flora of the hair coat of Persian squirrel (*Sciurus anomalus*) with and without skin lesion in Tehran, Iran. *Journal de Mycologie Médicale*, v. 20, p. 21-25, 2010.

SAMARANAYAKE, Y. H.; DASSANAYAKE, R. S.; JAYATILAKE, J. A. M. S.; CHEUNG, B. P. K.; YAU, J. Y. Y.; YEUNG, K. W. S.; SAMARANAYAKE, L. P. Phospholipase B enzyme expression is not associated with other virulence attributes of *Candida albicans* isolates from patients with human immunodeficiency virus infection. *Journal of Medical Microbiology*, v. 54, p. 583-593, 2005.

SAMOUR, J. H.; NALDO, J. L. Diagnosis and therapeutic management of candidiasis in falcons in Saudi Arabia. *Journal of Avian Medicine and Surgery*, v. 16, p. 129-132, 2002.

SANTOS, G. O.; RIGOTTO, R. M. Possíveis impactos sobre o ambiente e a saúde humana decorrentes dos lixões inativos de Fortaleza (CE). *Health and Environment Journal*, v.9, p.55-62, 2008.

SCHUETZER-MUEHLBAUER, M.; WILLINGER, B.; EGNER, R.; ECKER, G. KUCHLER, K. Reversal of antifungal resistance mediated by ABC efflux pumps from *Candida albicans* functionally expressed in yeast. *International Journal of Antimicrobial Agents*, v. 22, p. 291-300, 2003.

SHOKRI, H.; KHOSRAVI, A.; SHARIFZADEH, A.; TOOTIAN, Z. Isolation and identification of yeast flora from genital tract in healthy female camels (*Camelus dromedarius*). *Veterinary Microbiology*, v.144, p.183–186, 2010.

SIDRIM, J. J. C.; ROCHA, M. F. G. Candidíase. In: SIDRIM, J. J. C.; ROCHA, M. F. G. *Micologia médica à luz de autores contemporâneos*. Rio de Janeiro: Guanabara Koogan, 2004. cap. 25, p. 265-274.

SIDRIM, J. J. C.; CASTELO-BRANCO, D. S. C. M.; BRILHANTE, R. S. N.; SOARES, G. D. P.; CORDEIRO, R. A.; MONTEIRO, A. J.; ROCHA, M. F. G. *Candida* species isolated from the gastrointestinal tract of cockatiels (*Nymphicus hollandicus*): *In vitro* antifungal

susceptibility profile and phospholipase activity. *Veterinary Microbiology*, v. 145, p.324-328, 2010.

SIIKALA, E.; RAUTEMAA, R.; RICHARDSON, M.; SAXEN, H.; BOWYER, P.; SANGLARD, D. Persistent *Candida albicans* colonization and molecular mechanisms of azole resistance in autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED) patients. *Journal of Antimicrobial and Chemotherapy*, v. 65, p. 2505-2513, 2010.

SIMPSON, V. R.; DAVISON, N. J.; BORMAN, A. M.; LINTON, C. J.; EVEREST, D. Fatal candidiasis in a wild red squirrel (*Sciurus vulgaris*). *The Veterinary Record*, v. 164, p. 342-344, 2009.

SKORIC, M.; FICTUM, P.; SLANA, I.; KRIZ, P.; PAVLIK, I. A case of systemic mycosis in a Hovawart dog due to *Candida albicans. Veterinarni Medicina, v.* 56, p. 260-264, 2011.

SNELDERS, E.; HUISINT, R. A. G.; RIJS, A. J. M. M.; KEMA, G. H. J.; MELCHERS, W. J. G.; VERWEIJ, P. E. Possible environmental origin of resistance of *Aspergillus fumigatus*. *Applied of Environmetal Microbiology*, v.75, p.4053-4057, 2009.

SOUZA, W. A.; SIQUEIRA, A. M. Ocorrência de *Candida albicans* em intestinos de bovinos Occurrence of *Candida albicans* in bovine intestines. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, v.55, p. 262-265, 2003.

SPANAMBERG, A.; WÜNDER-JR., E. A.; PEREIRA, D. I. B.; ARGENTA, J.; SANCHES, E. M. C.; VALENTE, P.; FERREIRO, L. Diversity of yeasts from bovine mastitis in Southern Brazil. *Revista Iberoamericana de Micologia*, v. 25, p. 154-156, 2008.

TALBOT, N. J. 2001. Nucleic acid isolation and analysis. In: TALBOT, N. *Molecular and Cellular Biology of Filamentous Fungi*. New York, Oxford University Press, pp.23-31.

TAMURA, N. K.; NEGRI, M. F. N.; BONASSOLI, L. A.; SVIDZINSKI, T. I. E. Fatores de virulência de *Candida* spp. isoladas de cateteres venosos e mãos de servidores hospitalares. *Revista da Sociedade Brasileira de Medicina Tropical*, v. 40, p. 91-93, 2007.

TAKAHASHI, H.; UEDA, K.; ITANO, E. N.; YANAGISAWA, M.; MURATA, Y.; MURATA, M.; YAGUCHI, T.; MURAKAMI, M.; KAMEI, K.; INOMATA, T.; MIYAHARA, H.; SANO, A.; UCHIDA, S. *Candida albicans* and *C. tropicalis* isolates from

the expired breathes of captive dolphins and their environments in an aquarium. *Veterinary Medicine International*, v. 2010, p. 1-12, 2010.

TAWARA, S.; IKEDA, F.; MAKI, K.; MORISHITA, Y.; OTOMO, K.; TERATANI, N.; GOTO, T.; TOMISHIMA, M.; OHKI, H.; YAMADA, A.; KAWABATA, K.; TAKASUGI, H.; SAKANE, K.; TANAKA, H.; MATSUMOTO, F.; KUWAHARA, S. *In vitro* activities of a new lipopeptide antifungal agent, FK463, against a variety of clinically important fungi. *Antimicrobial and Agents Chemotherapy*, v.44, p.57-56, 2000.

TOHIDPOUR, A.; PEERAYEH, S. N.; MEHRABADI, J. F.; YAZDI, H. R. Determination of the efflux pump-mediated resistance prevalence in pseudomonas aeruginosa, using an efflux pump inhibitor. *Current Microbiology*, v.59, p.352–355, 2009;

VANDEPUTTE, P.; LARCHER, G.; BERGÈS, T.; RENIER, G.; CHABASSE, D.; BOUCHARA, J. P. Mechanisms of azole resistance in a clinical isolate of *Candida tropicalis*. *Antimicrobial Agents and Chemotherapy*, v. 49, p. 4608-4615, 2005.

VELASCO, M. C. Candidiasis and cryptococcosis in birds. *Seminars in Avian and Exotic Pet Medicine*, v. 9, p. 75-81, 2000.

VIEIRA, R. G.; ACQUA-COUTINHO, S. D. Phenotypical characterization of *Candida* spp. isolated from crop of parrots (*Amazona* spp.). *Pesquisa Veterinária Brasileira*, v. 29, p. 452-456, 2009.

VOGEL, M., HARTMANN, T., KOBERLE, M., TREIBER, M., AUTENRIETH, I. B., SCHUMACHER, U. K. Rifampicin induces *MDR1* expression in *Candida albicans*. *Journal of Antimicrobial Chemotherapy*, v. 61, 541–547, 2008.

WON, T. H.; PARK, S. Y.; KIM, B. S.; SEO, P. S.; PARK, S. D. Levamisole monotherapy for oral lichen planus. *Annals of Dermatology*, v. 21, p. 250-254, 2009.

WROBEL, L.; WHITTINGTON, J. K.; PUJOL, C.; OH, S. H.; RUIZ, M. O.; PFALLER, M. A.; DIEKEMA, D. J.; SOLL, D. R.; HOYER, L. L. Molecular phylogenetic analysis of a geographically and temporally matched set of *Candida albicans* isolates from Humans and Nonmigratory Wildlife in Central Illinois. *Eukaryotic Cell* v. 7, p. 1475–1486, 2008.

ZENG, X.; HOU, X.; WANG, Z.; JIANG, L.; XIONG, C; ZHOU, M.; CHEN, Q. Carriage rate and virulence attributes of oral *Candida albicans* isolates from patients with oral lichen planus: a study in an ethnic Chinese cohort. *Mycoses*, v. 52, p. 161-165, 2008.

ANEXO 1

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RESEARCH ARTICLE

Yeasts from *Macrobrachium amazonicum*: a focus on antifungal susceptibility and virulence factors of *Candida* spp.

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Yeast diversity; antifungal susceptibility; *Macrobrachium amazonicum*; virulence factors.

Abstract

In the present study, it was sought to compare yeast microbiota of wild and captive *Macrobrachium amazonicum* and evaluate the antifungal susceptibility and production of virulence factors by the recovered isolates of *Candida* spp. Additionally, cultivation water was monitored for the presence of fungi. Overall, 26 yeast isolates belonging to three genera and seven species were obtained, out of which 24 were *Candida* spp., with *Candida famata* as the most prevalent species for both wild and captive prawns. From cultivation water, 28 isolates of filamentous fungi were obtained, with *Penicillium* spp., *Cladosporium* spp. and *Aspergillus* spp. as the most frequent genera. Eight out of 24 *Candida* spp. isolates were resistant to azole derivatives, out of which four were recovered from wild-harvested prawns. As for production of virulence factors, three (12.5%) and eight (33.3%) isolates presented phospholipase and protease activity, respectively. This is the first comparative study between wild and captive prawns and the first report on yeast microbiota of *M. amazonicum*. The most relevant finding was the high percentage of resistant *Candida* spp., including from wild individuals, which suggests the occurrence of an environmental imbalance in the area where these prawns were captured.

Introduction

Macrobrachium amazonicum is a continental prawn (Martin & Davis, 2001) that is widely distributed in South America, from the Orenoco basin, through the Amazon River, to the Paraguay basin (Holthuis, 1952). Among all Brazilian native species, the Amazon river prawn (*M. amazonicum*) is the preferred one for cultivation, due to its rapid growth and easy maintenance in captivity (Moraes-Valenti & Valenti, 2010).

There are reports that emphasize the importance of yeasts and filamentous fungi for aquaculture, as pathogens of different groups of economic interest (Gatesoupe, 2007). *Candida* species represent the greatest number of isolates from prawn farming, with *Candida sake* as the most common one (Johnson & Bueno, 2000). Additionally, yeasts have been found in water and sediment from lakes and ponds inhabited by these crustaceans (Lu *et al.*, 1998).

Potentially pathogenic yeast species have been isolated from freshwater environments (Medeiros *et al.*, 2008) and animals (Brito *et al.*, 2009; Brilhante *et al.*, 2010). It is noteworthy that among these fungi, resistant strains have also been found. In a research work on lake water from Southeastern Brazil, resistance phenomena to itraconazole and amphotericin B in 50% of the isolated yeasts and 6% of the isolated *Candida* spp., respectively, were observed (Medeiros *et al.*, 2008). Additionally, the occurrence of resistance to ketoconazole, fluconazole and itraconazole in *Candida albicans* and *Candida tropicalis* obtained from dogs (Brito *et al.*, 2009) and to fluconazole and itraconazole among *C. albicans* recovered from cockatiels (*Nymphicus hollandicus*) (Sidrim *et al.*, 2010) was observed.

Until now, no studies comparing microbiota composition, antifungal susceptibility profile and virulence factors of yeasts recovered from *M. amazonicum* from natural

environment and captivity have been performed. Therefore, this study sought to compare yeast microbiota of captive and wild *M. amazonicum* and evaluate *in vitro* antifungal susceptibility and phospholipase and protease activities of recovered *Candida* spp. In addition, the presence of fungi in cultivation water was investigated.

Materials and methods

Wild-harvested prawns

Adults of *M. amazonicum* were harvested from Catu Lake (03°49′44,8″S and 38°29′9″W), in the municipality of Aquiraz, 35 km from Fortaleza, Ceará, Northeastern Brazil. The animals were captured by covos, a traditional trap used to capture this species of prawn, and were properly taken to Laboratory of Crustacean Culture of the State University of Ceará, where healthy individuals, presenting all thoracic and abdominal appendices, were randomly selected for this study.

Captive prawns

Experiment set-up and obtention of larvae

Larvae, juveniles and adults *M. amazonicum* that were cultivated at the Laboratory of Carciniculture of the State University of Ceará were used in this study. For the obtention of larvae, ovigerous females, in good health conditions, presenting all thoracic and abdominal appendices and eggs at the final stage of embryonary development, were used (Valenti *et al.*, 1998). These females were collected from Catu Lake, as described for wild-harvested prawns, and then maintained in a 60-L glass fiber tank, containing water with salinity of 4000 mg L^{-1}, under controlled temperature and abundant aeration, to which a biofilter was attached. These females were not fed while they were kept in the eclosion tank.

Set-up and maintenance of larviculture

Newly hatched larvae of *M. amazonicum* were stored in three rectangular tanks, each with a capacity of 70 L, filled with 60 L of water with different salinity, equipped with closed water circulation and heating systems. During larviculture, the experiments were performed in three different tanks, each one of them with different salinities: 2000, 4000 and 6000 mg L^{-1} for tanks T1, T2 and T3, respectively. A density of 20 larvae L^{-1} was used. Water temperature, pH and ammonia and nitrite concentrations were monitored daily.

Larvae were fed *ad libitum* with *Artemia* sp. nauplii, in the morning, from stages II to IX. Starting at stage V, larvae were also fed in the afternoon. After observing the first post-larva, polyvinyl chloride pipe substrates were placed into the tanks, in order to avoid cannibalism.

Larval health

Every 3 days, four larvae of *M. amazonicum* were taken from each tank and placed into a clock glass, containing water from the respective salinity, in order to observe larval stage and larval health status. Such evaluation was based on the criteria described previously for *Macrobrachium rosenbergii* larvae (Tayamen & Brown, 1999). Briefly, natatory behavior, presence of parasites and/or necrosis, intestinal conditions, pigmentation and phototaxis were evaluated.

Set-up and maintenance of grow-out

The post-larvae obtained during larviculture were stored in three different tanks, T4, T5 and T6, which contained the animals from the tanks with salinities of 2000 mg L^{-1} (T1), 4000 mg L^{-1} (T2) and 6000 mg L^{-1} (T3), respectively. The new tanks were circular, with a capacity of 400 L, filled to 360 L, equipped with closed water circulation and heating systems and populated at a density of 2 post-larvae L^{-1}. Only fresh water was used in the grow-out tanks, after filtering by activated charcoal filter. Water temperature, pH and ammonia and nitrite concentrations were monitored daily.

During 3 weeks, post-larvae were fed *ad libitum* with *Artemia* sp. nauplii and pelleted ration (Coyle *et al.*, 2010) only in the morning. After this period, an all-ration diet was instituted and offered in the morning and in the afternoon.

Collection of biological material for fungal isolation

After randomly selecting healthy wild adults from Catu Lake, the digestive tracts of 10 individuals were removed by making a dorsal transverse incision, and were placed in sterile slants containing sterile saline and were treated as one single sample. Overall, 18 collections were performed, with a total of 180 adult prawns.

After starting larviculture, 10 larvae from each tank (T1, T2 and T3) were collected, once a week, for 3 weeks, totaling 30 larvae per tank. After collection, larvae were ground and suspended in 1 mL of sterile saline (NaCl 0.9%). During the grow-out phase, 10 individuals (juveniles and/or adults) were collected, weekly, from each tank (T4, T5 and T6) and were treated as described for larvae, until reaching 2 cm in length. When the cultivated animals were bigger than 2 cm, their digestive tracts were removed, as described for wild-harvested adults. Overall, 15 collections were performed during this phase, with a total of 150 individuals per tank. After each collection, microbiological processing was immediately performed, as described in the following section.

Water samples from cultivation tanks were collected with 5 mL syringes. Aliquots of water were obtained from different regions of each tank (bottom, substrate, surface and

walls). Then, samples were taken to the laboratory, where they were homogenized in vortex and an aliquot of 100 μL was streaked onto microbiological media. All samples from animal and water were taken to the Specialized Medical Mycology Center (CEMM) of Federal University of Ceará, in Fortaleza, Ceará, Brazil.

Microbiological processing

Yeast isolation

For each sample, two culture media were used for primary isolation: 2% Sabouraud agar with chloramphenicol (0.5 g L^{-1}), and birdseed (*Guizotia abyssinica*) agar. Larvae and post-larvae suspensions were streaked onto the media, using a microbiological loop.

Digestive tracts were opened and homogenized in sterile Petri dishes, and approximately 1 g was added to a saline solution (NaCl 0.9%) containing chloramphenicol (0.4 g L^{-1}). The suspension was homogenized in a vortex for 3 min, and then left to decant for 30 min at 25 °C. Afterwards, aliquots of 100 μL from the supernatant of each sample were streaked onto both media (Brilhante *et al.*, 2010). Petri dishes containing the cultured media were incubated at 25 °C for 10 days, and were observed daily.

Identification

Initially, colonies that were suggestive of yeasts were observed microscopically (× 40), after Gram staining, in order to verify the presence of blastoconidia, hyphae or pseudohyphae, as well as to discard the occurrence of bacterial contamination. Then, yeasts were identified by performing specific morphological and biochemical tests and, when necessary, through VITEK 2 (bioMérieux®) (Brilhante *et al.*, 2010).

Briefly, identification of *Candida* species was based on phenotypical characteristics, such as macromorphology, colony observation, and micromorphology, after growth on Cornmeal-Tween 80 agar. Additionally, biochemical tests were performed, such as carbohydrate and nitrogen assimilation and urease production (De Hoog *et al.*, 2002). All *Candida* spp. isolates were grown on chromogenic medium (HiCrome Candida Differential Agar, HiMedia Laboratories, Mumbai, India), for the identification of mixed colonies (Brilhante *et al.*, 2010).

Cryptococcus spp. isolates were initially grown onto cornmeal Tween-80 agar and on Christensen's urea agar for microscopic and biochemical evaluation, which suggested the genus. Afterwards, an automated analysis was performed using VITEK 2 (bioMérieux®) in order to determine the species.

For *Rhodotorula* spp., colonies were initially identified based on their color. Then, the microorganism was submitted to urease production tests and grown on 2% malt extract agar for morphologic evaluation. Sugar assimilation tests were performed for each isolate and these were crucial for species identification (De Hoog *et al.*, 2000).

Identification of fungi from cultivation water

In order to identify filamentous fungi obtained from cultivation water, slide cultures on potato dextrose agar were performed. Micromorphological analysis was interpreted according to the identification keys (De Hoog *et al.*, 2000). Additionally, yeasts that were obtained from water were identified as described above.

In vitro antifungal susceptibility test

Twenty-four isolates of *Candida* spp. were tested: three *C. albicans* (one from larvae, one from the digestive tract of captive adults and another from the digestive tract of wild-harvested adults), three *C. tropicalis* (one from the digestive tract of captive adults and two from the digestive tract of wild-harvested adults), four *Candida parapsilosis* (three from the digestive tract of captive adults and one from the digestive tract of wild-harvested adults), 10 *Candida famata* (two from the digestive tract of captive adults, two from the cultivation water and six from the digestive tract of wild-harvested adults) and four *Candida guilliermondii* (one from the cultivation water and three from the digestive tract of wild-harvested adults).

The minimum inhibitory concentration (MIC) for these microorganisms was determined by a broth microdilution method as described by Clinical and Laboratory Standard Institute (CLSI, 2002) and in other researches of our group (Brito *et al.*, 2009; Sidrim *et al.*, 2010). As quality control for each test performed, *C. parapsilosis* ATCC 22019 was included. The strains were tested against four drugs: amphotericin B, caspofungin, itraconazole and fluconazole. Stock solutions of amphotericin B (Sigma Chemical Corporation) and itraconazole (Janssen Pharmaceutica, Belgium) were prepared with dimethyl sulfoxide and caspofungin (Merck Sharp & Dohme, Brazil) and fluconazole (Pfizer, Brazil) were diluted with distilled water.

Final concentrations of drugs (amphotericin B, caspofungin, itraconazole and fluconazole) were obtained according to some studies (CLSI, 2002; Pfaller *et al.*, 2006; Brito *et al.*, 2009). All drugs were diluted and resuspended in RPMI 1640 (HiMedia Laboratories). The concentration range tested for amphotericin B, caspofungin and itraconazole was 0.03125–16 μg mL^{-1} and for fluconazole was 0.125–64 μg mL^{-1} (Pfaller *et al.*, 2006; Brito *et al.*, 2009). The criteria for resistance and sensitivity were established according to some authors (CLSI, 2002; Pfaller *et al.*, 2006). Isolates with MICs > 1, ≥2, ≥1 and ≥64 μg mL^{-1} were

considered resistant to amphotericin B, caspofungin, itraconazole and fluconazole, respectively.

Inocula of all tested isolates were prepared from 1-day-old cultures grown on potato dextrose agar at 35 °C. Sterile 0.9% saline (5 mL) was added to sterile glass slants and a sample of the colony was added to the saline solution, adjusting its concentration to 0.5 on McFarland Scale (CLSI, 2002). Afterwards, inocula were diluted 1 : 100 and then 1 : 20, in RPMI 1640 medium, with L-glutamine (HiMedia Laboratories), buffered to pH 7 with 0.165 M morpholinepropanesulfonic acid. The final concentration of the inocula was $0.5–2.5 \times 10^3$ cells mL^{-1} (CLSI, 2002; Brito *et al.*, 2009).

Susceptibility testing was performed on 96-well microdilution trays, which were properly prepared and incubated at 35 °C, for 48 h (Brito *et al.*, 2009). For each isolate, drug-free and yeast-free controls were included and all the isolates were tested in duplicate. For the azole derivatives and caspofungin, the MIC was defined as the lowest drug concentration inhibiting 80% growth when compared with the growth in the control well, and for amphotericin B the MIC was the lowest concentration at which no growth was observed (CLSI, 2002; Brito *et al.*, 2009; Sidrim *et al.*, 2010).

Minimum fungicidal concentration (MFC)

After obtaining MIC values, microdilution trays were agitated in order to homogenize the inocula and 100-μL aliquots of each isolate, from three consecutive wells with increasing drug concentration, starting at the MIC, were subcultured into slants containing 2% Sabouraud dextrose agar, which were incubated for 48 h at 35 °C. The MFC was defined as the lowest drug concentration at which subcultures presented negative results or produced less than three colonies, indicating the death of $> 99\%$ of the original inoculum (Tawara *et al.*, 2000).

Phospholipase production

The same isolates that were tested for antifungal susceptibility were evaluated for phospholipase activity. The test was performed according to the previously described methodology (Price *et al.*, 1982), with some modifications. Briefly, the medium used was 2% Sabouraud dextrose agar, to which 1 mol L^{-1} sodium chloride, 0.05 mol L^{-1} calcium chloride and 8% sterile egg yolk emulsion, at a concentration of 30%, were added. The emulsion was heated up to 40 °C and incorporated into the sterile medium, after it reached a temperature of 50 °C. Then, the medium was poured into 90-mm Petri dishes, forming a 4-mm film. Yeast inocula were prepared in sterile saline at a final concentration of 4 on McFarland scale. A 5-μL drop of each inoculum was placed on a 5-mm sterilized filter paper disk, which was then placed on the agar. The plates were incubated at 35 °C, for 7 days (Sidrim *et al.*, 2010).

Phospholipase activity (Pz) was determined by calculating the ratio between the diameter of the fungal colony and the total diameter, including the colony and the precipitation zone. Thus, when $Pz = 1$, the isolate is phospholipase negative; when $1 > Pz \geq 0.64$, the isolate is positive for phospholipase activity; and when $Pz < 0.64$, the isolate is strongly positive for this enzyme (Sidrim *et al.*, 2010).

Protease production

The 24 isolates of *Candida* spp. were also screened for protease production. The protease test was performed according to a previously described methodology (Charney & Tomarelli, 1947; Cenci *et al.*, 2008) with modifications. Briefly, yeast inocula were prepared from 1-day-old cultures in a sterile saline solution, reaching a final concentration of 4 on McFarland scale. Then, the inocula were cultured in RPMI medium in a 1 : 1 dilution, by adding 2 mL of each inoculum to 2 mL of RPMI. After 48 h of incubation in a shaker at 150 r.p.m., the inocula were centrifuged at 805 ***g*** for 15 min, and the supernatant was divided into three slants containing 1 mL each. The content from one of the slants was considered the reference substance (blank) and the content from the other two slants was used for testing in duplicate. To the blank content, 1 mL of trichloroacetic acid and 1 mL of azoalbumine were added, while only 1 mL of azoalbumine was added to the other slants. All slants were incubated in 37 °C bath for 48 h, after which the reaction was stopped by adding 1 mL of trichloroacetic acid to the tested slants. Then, the tubes were centrifuged for 30 min at 805 ***g***, and 2 mL of the supernatant were added to 2 mL of 5% NAOH for posterior spectrophotometric analysis at an absorbance of 530 nm.

Statistical analysis

In order to compare yeast prevalence, Fisher's exact test was applied. Pearson's correlation coefficient was used to calculate the correlation between MICs and MFCs. Paired-samples Student's *t*-test was used to compare MICs with MFCs. For all cases, a significance level of 5% was adopted for significant conclusions.

Results

From 126 samples of wild-harvested adults, captive animals and tank water, 26 yeast isolates belonging to three genera and seven species were recovered. Fourteen (54%), eight (31%) and four (15%) isolates were obtained from wild-harvested prawns, captive prawns and cultivation water, respectively (Table 1).

From the digestive tract of wild-harvested adult prawns, 14 (14/26; 53.85%) yeast isolates were recovered: one *C. albicans*,

Table 1. Yeast species isolated from *Macrobrachium amazonicum* and water from cultivation

	Collection site													
	Wild prawns		Captive prawns						Water				Total	
			Larvae		Juvenile and adult				Larviculture		Grow-out			
			T2		T4		T5		T2		T6			
Yeast species	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Candida albicans	1	3.85	1	3.85	–	–	1	3.85	–	–	–	–	3	11.54
Candida famata	6	23.08	–	–	1	3.85	1	3.85	1	3.85	1	3.85	10	38.46
Candida guilliermondii	3	11.54	–	–	–	–	–	–	1	3.85	–	–	4	15.38
Candida parapsilosis	1	3.85	–	–	–	–	3	11.54	–	–	–	–	4	15.38
Candida tropicalis	2	7.69	–	–	–	–	1	3.85	–	–	–	–	3	11.54
Cryptococcus laurentii	1	3.85	–	–	–	–	–	–	–	–	–	–	1	3.85
Rhodotorula mucilaginosa	–	–	–	–	–	–	–	–	1	3.85	–	–	1	3.85
Total	14	53.84	1	3.85	1	3.85	6	23.07	3	11.54	1	3.85	26	100

T2, larviculture tank (salinity 4 mg L^{-1}); T4, grow-out tank (fresh water) corresponding to T1 (salinity 2 mg L^{-1}); T5, grow-out tank (fresh water) corresponding to T2 (salinity 4 mg L^{-1}); T6, grow-out tank (fresh water) corresponding to T3 (salinity 6 mg L^{-1}).

Table 2. Filamentous fungus species isolated from cultivation tanks of *Macrobrachium Amazonicum*

	Collection site											
	Larviculture water				Grow-out water						Total	
	T2		T3		T4		T5		T6			
Species	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Penicillium sp.	1	3.57	1	3.57	2	7.15	–	–	3	10.71	7	25.00
Penicillium decumbens	–	–	–	–	1	3.57	1	3.57	1	3.57	3	10.71
Penicillium chrysogenum	–	–	–	–	1	3.57	–	–	–	–	1	3.57
Penicillium griseofulvum	–	–	–	–	1	3.57	1	3.57	–	–	2	7.15
Cladosporium sp.	1	3.57	–	–	2	7.15	1	3.57	–	–	4	14.29
Cladosporium sphaerospermum	–	–	–	–	–	–	1	3.57	–	–	1	3.57
Cladosporium cladosporioides	–	–	–	–	1	3.57	–	–	1	3.57	2	7.15
Aspergillus sp.	–	–	–	–	1	3.57	1	3.57	1	3.57	3	10.71
Aspergillus niger	–	–	–	–	–	–	1	3.57	–	–	1	3.57
Hortae werneckii	2	7.14	1	3.57	–	–	–	–	–	–	3	10.71
Mucor racemosus	–	–	–	–	0	0	0	0	1	3.57	1	3.57
Total	4	14.28	2	7.15	9	32.15	6	21.42	7	25.00	28	100

T2, larviculture tank (salinity 4 mg L^{-1}); T3, larviculture tank (salinity 6 mg L^{-1}); T4, grow-out tank (fresh water) corresponding to T1 (salinity 2 mg L^{-1}); T5, grow-out tank (fresh water) corresponding to T2 (salinity 4 mg L^{-1}); T6, grow-out tank (fresh water) corresponding to T3 (salinity 6 mg L^{-1}).

six *C. famata*, two *C. tropicalis*, one *C. parapsilosis*, three *C. guilliermondii* and one *Cryptococcus laurentii* (Table 1).

Out of eight yeast isolates from captive prawns, one was obtained from larvae (1/26; 3.85% – *C. albicans*) and no isolates were recovered from post-larvae. On the other hand, seven isolates were obtained from the digestive tract of adult prawns (7/26; 26.92% – one *C. albicans*, two *C. famata*, one *C. tropicalis* and three *C. parapsilosis*) (Table 1). *Candida famata* ($P = 0.0311$) and *C. guilliermondii* ($P = 0.0599$) were more frequently isolated from wild-harvested prawns, when compared with those from captivity.

No yeast isolates were recovered from larviculture water with salinities of 2000 and 6000 mg L^{-1} (T1 and T3, respectively) or from grow-out tanks T4 and T5. Three isolates (3/26; 11.54% – one *C. famata*, one *C. guilliermondii* and one *Rhodotorula mucilaginosa*) were obtained from water with a salinity of 4000 mg L^{-1}. (T2) and one water sample from grow-out tank T6 was positive for yeast growth (1/26; 3.85% – *C. famata*) (Table 1).

It was possible to recover filamentous fungi from 42.6% (23/54) of cultivation water samples. These fungi belonged to five genera, with a total of 28 isolates (Table 2). Four (4/28; 14.29%) and two (2/28; 7.14%) isolates were obtained from larviculture tanks T2 and T3, respectively. No filamentous fungi were recovered from the larviculture tank with a salinity of 2000 mg L^{-1} (T1). Nine (9/28; 32%), six (6/28;

Table 3. MIC and MFC distribution of amphotericin B, caspofungin, itraconazole and fluconazole against 24 isolates of *Candida* spp.

Candida spp.		Amphotericin B (μg mL^{-1})		Caspofungin (μg mL^{-1})		Itraconazole (μg mL^{-1})		Fluconazole (μg mL^{-1})	
	n	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
C. albicans	3	0.125 (1)*	0.125 (1)	0.03125 (2)	0.03125 (1)	≥16 (3)	≥16 (3)	≥64 (3)	≥64 (3)
		0.5 (2)	0.5 (2)	0.0625 (1)	0.0625 (1)				
					0.25 (1)				
C. famata	10	0.03125 (5)	0.03125 (5)	0.03125 (1)	0.03125 (1)	0.03125 (2)	0.03125 (1)	0.5 (2)	2 (2)
		0.0625 (3)	0.0625 (3)	0.0625 (2)	0.0625 (2)	0.0625 (3)	0.0625 (1)	2 (3)	4 (1)
		0.125 (1)	0.125 (1)	0.125 (1)	0.125 (1)	0.125 (2)	0.125 (2)	4 (1)	16 (2)
		0.5 (1)	0.5 (1)	0.25 (2)	0.25 (1)	0.5 (1)	0.25 (1)	8 (1)	32 (2)
				0.5 (4)	1 (1)	≥16 (2)	0.5 (3)	32 (1)	≥64 (3)
					2 (3)		≥16 (2)	≥64 (2)	
					4 (1)				
C. parapsilosis	4	0.0625 (1)	0.0625 (1)	0.25 (1)	2 (2)	0.03125 (1)	0.625 (1)	8 (2)	4 (1)
		0.125 (2)	0.125 (2)	0.5 (1)	4 (1)	0.0625 (1)	0.5 (1)	4 (2)	8 (2)
		0.25 (1)	0.25 (1)	1 (2)	8 (1)	0.5 (1)	2 (1)		32 (1)
						≥16 (1)	≥16 (1)		
C. tropicalis	3	0.125 (3)	0.125 (3)	0.125 (2)	0.5 (1)	0.03125 (2)	0.0625 (2)	8 (2)	8 (1)
				0.5(1)	1 (1)	4 (1)	≥16 (1)	≥64 (1)	32 (1)
					2 (1)				≥64 (1)
C. guilliermondii	4	0.03125 (1)	0.03125 (1)	0.03125 (2)	0.03125 (1)	0.03125 (3)	0.03125 (1)	0.5 (1)	4 (1)
		0.0625 (2)	0.625 (2)	0.0625 (1)	0.25 (1)	≥16 (1)	0.0625 (1)	1 (1)	8 (2)
		0.25 (1)	0.25 (1)	1 (1)	1 (1)		0.125 (1)	2 (1)	16 (1)
					4 (1)		≥16 (1)	8(1)	

*Represents the number of isolates for each indicated MIC and MFC.

21.43%) and seven (7/28; 25%) isolates were obtained from water samples from grow-out tanks T4, T5 and T6, respectively.

Penicillium sp. was the most isolated genus (13/28; 46.42%), followed by *Cladosporium* sp. (7/28; 25%) and *Aspergillus* sp. (4/28; 14.29%). Other obtained isolates were *Hortaea werneckii* (3/28; 10.71%) and *Mucor* sp. (1/28; 3.57%) (Table 2).

Concerning *in vitro* antifungal susceptibility tests of *Candida* spp., the MIC values for all tested isolates are described in Table 3. Briefly, for *C. famata* (10/24), the MIC for amphotericin B varied from 0.03125 to 0.5 μg mL^{-1}, and for caspofungin, itraconazole and fluconazole, MICs ranged from 0.03125 to 0.5 μg mL^{-1}, 0.03125 to ≥16 μg mL^{-1} and 0.5 to ≥64 μg mL^{-1}, respectively. For *C. parapsilosis* (4/24), MIC for amphotericin B, caspofungin, itraconazole and fluconazole ranged from 0.0625 to 0.25 μg mL^{-1}, 0.25 to 1 μg mL^{-1}, 0.03125 to ≥16 μg mL^{-1} and 4 to 8 μg mL^{-1}, respectively. For *C. guilliermondii* (4/24), MICs varied from 0.03125 to 0.25 μg mL^{-1}, 0.03125 to 1 μg mL^{-1}, 0.03125 to ≥16 μg mL^{-1} and 0.5 to 8 μg mL^{-1} for amphotericin B, caspofungin, itraconazole and fluconazole, respectively. For *C. tropicalis* (3/24), the MIC value for amphotericin B was 0.125 μg mL^{-1}, while those for caspofungin, itraconazole and fluconazole ranged from 0.125 to 0.5 μg mL^{-1}, 0.03125 to 4 μg mL^{-1} and 8 to ≥64 μg mL^{-1}, respectively. Finally, for *C. albicans* (3/24), MIC values for amphotericin B and caspofungin varied from 0.125 to 0.5 μg mL^{-1} and 0.03125 to 0.0625 μg mL^{-1}, respectively, while those for itraconazole and fluconazole were ≥16 and ≥64 μg mL^{-1}, respectively. A positive correlation was observed when comparing MICs for amphotericin B and itraconazole ($P = 0.0005$), amphotericin B and fluconazole ($P = 0.0020$), and fluconazole and itraconazole ($P = 0.0007$), but not for caspofungin, when compared with the other tested drugs.

MFC values for all *Candida* spp. isolates varied from 0.03125 to 0.5 μg mL^{-1}, 0.03125 to 8 μg mL^{-1}, 0.03125 to ≥16 μg mL^{-1} and 2 to ≥64 μg mL^{-1} for amphotericin B, caspofungin, itraconazole and fluconazole, respectively (Table 3). A positive correlation was also observed when comparing MFCs for amphotericin B and itraconazole ($P = 0.0001$), amphotericin B and fluconazole ($P = 0.0104$), and fluconazole and itraconazole ($P = 0.0013$), but not for caspofungin, when compared with the other tested drugs. When comparing MIC and MFC, a positive correlation was observed for caspofungin ($P = 0.0000$), fluconazole ($P = 0.0000$) and itraconazole ($P = 0.0000$). For amphotericin B, MICs were equal to MFCs; for itraconazole, MICs and MFCs were not statistically different ($P = 0.1108$), and for caspofungin ($P = 0.0007$) and fluconazole ($P = 0.0013$), MICs and MFCs were statistically different.

Concerning phospholipase activity, only three (12.5%) isolates presented positive results (Pz < 1). The obtained Pz

values were 0.64, 0.67 and 0.71 for *C. famata*, *C. tropicalis* and *C. parapsilosis*, respectively. *Candida famata* was isolated from the larviculture water (T1) and *C. tropicalis* and *C. parapsilosis* were isolated from the digestive tract of captive adults (T5).

As for protease production, eight isolates presented positive results (8/24; 33.3%), which ranged from 1.0 to 27.0 U μL^{-1}. These isolates were four *C. famata* (4/10; 40%; one from T3 water and three from wild-harvested adults), two *C. albicans* (2/3; 66.7%; one from wild-harvested adults and one from captive adult from T5), one *C. parapsilosis* (1/4; 25%; from captive adult from T5) and one *C. guilliermondii* (1/4; 25%; from wild-harvested adult).

Discussion

The larval health index was satisfactory for all analyzed tanks during this experiment, with values > 1 (Tayamen & Brown, 1999). The water temperature varied from 28 to 30 °C and ammonia and nitrite concentrations were maintained at zero throughout the research, within ideal management conditions for *M. amazonicum* (Moraes-Valenti & Valenti, 2010).

Candida was the most isolated genus and *C. famata* the most frequently isolated species, with 10 isolates (38.46%), followed by *C. guilliermondii* (15.4%) and *C. parapsilosis* (15.4%). Other recovered species were *C. albicans*, *C. tropicalis*, *C. laurentii* and *R. mucilaginosa*. *Candida albicans* was recovered from larvae and adult prawns, but not from water, corroborating the generally accepted idea that ecological niches for this yeast species do not exist in the environment. Medeiros *et al.*, 2008 recovered several *Candida* species from water and sediment samples from two unpolluted lakes, but *C. albicans* was not recovered, supporting this idea.

Candida famata was the most commonly isolated species from wild-harvested adult prawns and cultivation water (42% and 50%, respectively). From the digestive tract of cultivated prawns, *C. tropicalis* and *C. famata* were the most frequently recovered species (28.6% each). The digestive tract of wild-harvested adult *M. amazonicum* from Catu Lake represented the type of sample where the highest number of species was found, followed by digestive tracts of cultivated animals, cultivation water and larvae. Considering that the intestinal microbiota of aquatic invertebrates resembles that of the environment where they are inserted (Hagler *et al.*, 1995; Kutty & Philip, 2008), this observation was expected, once environments under natural conditions are subjected to several influencing factors, including natural and anthropic ones, harboring a greater diversity of microorganisms (Medeiros *et al.*, 2008), when compared with environments under controlled conditions, commonly used in prawn farming.

In a study with *M. rosenbergii* cultivated in Taiwan, the greatest percentage of the isolates (86%) were represented by *Candida* species, out of which 70% were *C. sake* and 16% were *C. famata* (Lu *et al.*, 1998). However, 61% of the animals presented clinical alterations, which may explain the high prevalence of *C. sake*, a well-known pathogen of freshwater prawns. In our study, only healthy prawns were assessed and *C. sake* was not recovered, while *C. famata* was the most frequently isolated species, demonstrating the presence of this yeast species in these animals.

In our study, although the number of yeasts recovered from the cultivation water was small, *Candida* spp. were the predominantly isolated ones, similar to that observed by Lu *et al.*, 1998 and Leaño *et al.*, 2005 in studies with captive giant river prawns and tiger prawns, respectively. The water-sampling methodology used in this research differed from those mentioned by other authors (Lu *et al.*, 1998; Leaño *et al.*, 2005; Medeiros *et al.*, 2008), which could have accounted for the low recovery rate of yeasts, including the inability to obtain *C. albicans* from cultivation water. However, these authors studied the quality of water from natural sources or ponds, but not from tanks equipped with closed water circulation system, which justifies their need to work with larger volumes of water.

The three predominant genera of filamentous fungi found in cultivation water of this study, *Penicillium*, *Cladosporium* and *Aspergillus*, were also found in an investigation of filamentous fungi in cultivation water of tilapia and tiger prawns by Leaño *et al.*, 2005. *Penicillium* sp. and *Aspergillus* sp. were the predominant genera found by these authors, but *Cladosporium* sp. was isolated from only one sample of the tiger prawn cultivation. In contrast, *Cladosporium* sp. represented 25% of the isolates in our study, which probably reflects the environmental condition under which this research was conducted, considering that the species belonging to this genus, along with *Penicillium* spp. and *Aspergillus* spp., represent common airborne fungi in indoor and outdoor environments (Solomon *et al.*, 2006; Pantoja *et al.*, 2009; Miao *et al.*, 2010).

In our study, the fungus *H. werneckii* (3/28) was isolated from water samples from tanks T2 and T3 (salinities of 4000 and 6000 mg L^{-1}, respectively). This fungus inhabits tropical and subtropical regions (De Hoog *et al.*, 2000; Varga & Godoy, 2004) and it is the predominant species in hypersaline waters, acting as a saprobic microorganism in these environments (Gunde-Cimerman *et al.*, 2000; Mbata, 2008). *Hortaea werneckii* can inhabit a wide range of salinities, varying from 0 to 25 000 mg L^{-1}, but optimal growth occurs between 3000 and 7500 mg L^{-1} of NaCl (Petrovic *et al.*, 2002), which includes the salinity of the samples from which this fungus was isolated in our study.

Although some filamentous fungi are identified as pathogens for prawns, such as *Aspergillus* sp., their isolation was not harmful to the health of cultivated prawns in this study, considering that no clinical alterations were observed. Most

pathogenic fungi for prawns and shrimps belong to the microbiota of cultivation water and are secondary or opportunistic invaders. These microorganisms cause cultivation problems only when the animals are submitted to inadequate management conditions, which favor the dissemination of other diseases (Leaño *et al.*, 2005). Hence, the animals were kept under adequate management conditions

In this study, all *Candida* strains were susceptible to amphotericin B, as observed in other researches performed by our group (Brito *et al.*, 2009; Sidrim *et al.*, 2010). Eight out of 24 *Candida* spp. isolates (33.3%) were resistant to itraconazole and/or fluconazole, with particular attention given to the isolates of *C. albicans*, which were resistant to both drugs simultaneously. The MFC results obtained in our study were similar to those for clinical isolates of non-*albicans Candida* species (Tawara *et al.*, 2000).

Additionally, it is noteworthy that 28.6% (4/14) of the isolates of *Candida* spp. obtained from wild-harvested *M. amazonicum* were resistant to these azole derivatives. The observed resistance phenomenon to this class of drugs arose the curiosity to seek the causes that may be associated with this phenomenon in the environment. Catu Lake is a freshwater source that has been used for human consumption and supply, animal consumption, agriculture, industries and leisure activities. Considering that industrial wastes and pollutants are reported as causes of mutagenesis in yeasts (Keenan *et al.*, 2007) and that the occurrence of gene mutation (Feng *et al.*, 2010) may result in antifungal resistance, we believe that the resistance phenomenon observed in these *Candida* strains is related to anthropic activities developed in the studied area, such as pollution with industrial, agricultural and farming wastes.

As mentioned before, the intestinal microbiota of aquatic invertebrates is similar to that of the environment where they are inserted (Hagler *et al.*, 1995; Kutty & Philip, 2008); thus, yeasts isolated from the digestive tract of wild-harvested *M. amazonicum* may reflect the environmental conditions of Catu Lake. Based on this, crustaceans, including *M. amazonicum*, and fishes can be used as sentinels for the occurrence of resistant yeasts in the environment. Several animal species have been reported as sentinels for the occurrence of resistant bacteria in the wild, including predatory fishes (Blackburn *et al.*, 2010) and terrestrian mammals (Routman *et al.*, 1985; Costa *et al.*, 2008; Mariano *et al.*, 2009). Interestingly, all of these authors reported that the occurrence of resistant bacteria was associated with the close relationship between these wild animals or their environment and human beings and that water was the main vehicle for spreading chemical compounds involved in the development of resistance.

Classically, bacteria have been used as bioindicators of environmental imbalance related to pollution. However, more recently, the use of yeasts has been encouraged, especially because of the structural and molecular similarities between fungal and mammalian cells (Keenan *et al.*, 2007). Several pollutants are capable of altering gene expression or sequence composition (Wegrzyn & Czyz, 2003; Keenan *et al.*, 2007; Müller *et al.*, 2007). Considering that azole resistance is basically associated with changes in gene expression or in gene nucleotide sequence, the presence of pollutants in the water of this lake, including azole derivatives commonly used in agricultural practices, may be responsible for the observed resistance. Based on these observations, we propose the use of *M. amazonicum* and other aquatic species as sentinels for the occurrence of resistant yeasts in the environment and the use of the isolated yeast as bioindicators for the presence of pollutants in natural water sources.

Additionally, the risk of yeast infections occurring in humans should be considered when manipulating these animals or the water they inhabit, considering that potentially pathogenic species were isolated from these sources. Three species should be highlighted, *C. albicans*, *C. parapsilosis* and *C. tropicalis*, for being the most commonly involved in human candidiasis in Latin America (Palacio *et al.*, 2009). Besides, special attention must be given to *C. albicans*, considering that animals can represent a source of infection for humans (Edelmann *et al.*, 2005) and that the recovered isolates of this species were all azole resistant, which is not common among human strains (Kanafani & Perfect, 2008). Thus, studies on genetic diversity of this *Candida* species could help to elucidate whether or not the recovered isolates are commonly distributed among humans.

There are several researches concerning phospholipase activity of fungi isolated from humans (Fotedar & Al-Hedaithy, 2005; Zeng *et al.*, 2008). However, researches with yeasts isolated from animals are scarce (Sidrim *et al.*, 2010) and no reports have been found for yeasts from prawns or shrimps. For many years, it was believed that only *C. albicans* was able to produce phospholipase. However, now it is known that other species of *Candida* also produce this enzyme, usually in smaller amounts (Ghannoum, 2000), as demonstrated by our research. Paradoxically, the isolates of *C. albicans* obtained in this study did not present phospholipase activity, while 14.29% (3/21) of the non-*albicans Candida* species were positive for this enzyme.

Some studies have evaluated the protease activity of *Candida* species; however, in those researches, the strains were obtained from humans with candidiasis (Kantarcioglu & Yucel, 2002; Mohan & Ballal, 2008; Ombrella *et al.*, 2008). In our study, the production of protease by *Candida* spp. strains isolated from wild-harvested and captive crustaceans was demonstrated for the first time.

Finally, this work represented the first study on yeast microbiota of *M. amazonicum*, comparing wild and captive populations. Additionally, the most relevant finding was the

isolation of azole-resistant *Candida* spp. from wild-harvested animals, showing the role of these animals as sentinels for the occurrence of resistant yeasts in the natural environment.

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References

Blackburn JK, Mitchell MA, Blackburn MCH, Curtis A & Thompson BA (2010) Evidence of antibiotic resistance in free-swimming top-level marine predatory fishes. *J Zoo Wildlife Med* **41**: 7–16.

Brilhante RS, Castelo-Branco DSCM, Soares GD, Astete-Medrano DJ, Monteiro AJ, Cordeiro RA, Sidrim JJ & Rocha MF (2010) Characterization of the gastrointestinal yeast microbiota of cockatiels (*Nymphicus hollandicus*): a potential hazard to human health. *J Med Microbiol* **59**: 718–723.

Brito EHS, Fontenelle ROS, Brilhante RSN, Cordeiro RA, Monteiro AJ, Sidrim JJC & Rocha MFG (2009) The anatomical distribution and antimicrobial susceptibility of yeast species isolated from healthy dogs. *Vet J* **182**: 320–326.

Cenci E, Francisci D, Belfiori B, Pierucci S, Baldelli F, Bistoni F & Vecchiarelli A (2008) Tipranavir exhibits different efects on opportunistic pathogenic fungi. *J Infect* **56**: 58–64.

Charney J & Tomarelli RM (1947) A colorimetric method for the determination of the proteolytic of duodenal juice. *J Biol Chem* **23**: 501–505.

Costa D, Poeta P, Sáenz Y, Vinué L, Coelho AC, Matos M, Rojo-Bezares B, Rodrigues J & Torres C (2008) Mechanisms of antibiotic resistance in *Escherichia coli* isolates recovered from wild animals. *Microb Drug Resist* **14**: 71–77.

Coyle SD, Alston DE & Sampaio CMS (2010) Nursery systems and management. *Freshwater Prawns: Biology and Farming* (New MB, Valenti WC, Tidwell JH, D'abramo LR & Kutty MN, eds), pp. 108–126. Wiley-Blackwell, Oxford.

De Hoog GS, Guarro J, Gené J & Figueiras MJ (eds) (2000) *Atlas of Clinical Fungi*. Centraalbureau voor Schimmslcultures, The Nederlands.

De Hoog GS, Guarro J & Gené J (eds) (2002) *Atlas of Clinical Fungi*. Centraalbureau voor Schinmelculture/Universitat Rovira i Virgilli, Delf.

Edelmann A, Krüger M & Schmid J (2005) Genetic relationship between human and animal isolates of *Candida albicans*. *J Clin Microbiol* **43**: 6164–6166.

Feng L, Wan Z, Wang X, Li R & Liu W (2010) Relationship between antifungal resistance of fluconazole resistant *Candida albicans* and mutations in *ERG11* gene. *Chin Med J* **123**: 544–548.

Fotedar R & Al-Hedaithy SSA (2005) Comparison of phospholipase and proteinase activity in *Candida albicans* and *C. dubliniensis*. *Mycoses* **48**: 62–67.

Gatesoupe FJ (2007) Live yeasts in the gut: natural occurrence, dietary introduction, and their effects on fish health and development. *Aquaculture* **267**: 20–30.

Ghannoum MA (2000) Potential role of phospholipases in virulence and fungal pathogenesis. *Clin Microb Rev* **13**: 122–143.

Gunde-Cimerman N, Zalar P, De Hoog S & Plemenitas A (2000) Hypersaline waters in salterns – natural ecological niches for halophilic black yeasts. *FEMS Microbiol Ecol* **32**: 235–240.

Hagler AN, Mendonça-Hagler LC, Rosa CA & Morais PP (1995) Yeast as an example of microbial diversity in brazilian ecosystems. *Oecologia brasiliensis* **1**: 225–244.

Holthuis LB (1952) A general revision of the Palemonidae (Crustacea, Decapoda, Natantia) of the Americas. *Allan Hancock Foundation* **12**: 1–396.

Johnson SK & Bueno SLS (2000) Health management. *Freshwater Prawn Culture: The Farming of Macrobrachium Rosenbergii, Vol. 1* (New MB & Valenti WC, eds), pp. 18–40. Blackwell Science, London.

Kanafani ZA & Perfect JR (2008) Resistance to antifungal agents: mechanisms and clinical impact. *Clin Infect Dis* **46**: 120–128.

Kantarcioglu AS & Yucel A (2002) Phospholipase and protease activities in clinical Candida isolates with reference to the sources of strains. *Mycoses* **45**: 160–165.

Keenan PO, Knight AW, Billinton N, Cahill PA, Dalrymple IM, Hawkyard CJ, Stratton-Campbellc D & Walmsleya RM (2007) Clear and present danger? The use of a yeast biosensor to monitor changes in the toxicity of industrial effluents subjected to oxidative colour removal treatments. *J Environ Monitor* **9**: 1394–1401.

Kutty SN & Philip R (2008) Marine yeasts – a review. *Yeast* **25**: 465–483.

Leaño EM, Lio-Po GD, Nadong LA, Tirado AC, Sadaba RB & Guanzon NG Jr (2005) Mycoflora of the 'green water' culture system of tiger shrimp *Penaeus monodon* Fabricius. *Aquac Res* **36**: 1581–1587.

Lu CC, Tang KFJ & Chen SN (1998) Identification and genetic characterization of yeasts isolated from freshwater prawns, *Macrobrachium rosenbergii* de Man, in Taiwan. *J Fish Dis* **21**: 185–192.

Mariano V, McCrindle CME, Cenci-Goga B & Picard JA (2009) Case–control study to determine whether river water can spread tetracycline resistance to unexposed impala (*Aepyceros melampus*) in Kruger National Park (South Africa). *Appl Environ Microb* **75**: 113–118.

Martin JW & Davis GE (2001) An update classification of the recent Crustacea. *Nat Hist Mus Los Angeles* **39**: 1–124.

Mbata TI (2008) Isolation of fungi in hyper saline Dead Sea water. *Sudanese J Public Health* **3**: 170–172.

Medeiros AO, Kohler LM, Hamdan JS, Missagia BS, Barbosa FAR & Rosa CA (2008) Diversity and antifungal susceptibility of

yeasts from tropical freshwater environments in Southeastern Brazil. *Water Res* **42**: 3921–3929.

Miao Z, Chai T, Qi C, Cai Y, Liu J, Yuan W & Yao M (2010) Composition and variability of airborne fungi in an enclosed rabbit house in China. *Aerobiologia* **26**: 135–140.

Mohan V & Ballal M (2008) Proteinase and phospholipase activity as virulence factors in *Candida* species isolated from blood. *Rev Iberoam Micol* **25**: 208–210.

Moraes-Valenti P & Valenti WC (2010) Culture of the Amazon river prawn *Macrobrachium amazonicum*. *Freshwater Prawns: Biology and Farming* (New MB, Valenti WC, Tidwell JH, D'abramo LR & Kutty MN, eds), pp. 485–501. Wiley-Blackwell, Oxford.

Müller FMC, Staudigel A, Salvenmoser S, Tredup A, Miltenberger R & Herrmann JV (2007) Cross-resistance to medical and agricultural azole drugs in yeasts from the oropharynx of human immunodeficiency virus patients and from environmental bavarian vine grapes. *Antimicrob Agents Ch* **51**: 3014–3016.

National Committee for Clinical Laboratory Standards (2002) Reference method for broth dilution antifungal susceptibility testing of yeasts: approved standard. NCCLS, document M27-A2.

Ombrella AM, Racca L & Ramos L (2008) Actividades proteinasa y fosfolipasa de aislamientos de *Candida albicans* provenientes de secreciones vaginales con distintos valores de pH. *Rev Iberoam Micol* **25**: 12–16.

Palacio A, Villar J & Alhambra A (2009) Epidemiología de las candidiasis invasoras em población pediátrica y adulta. *Rev Iberoam Micol* **26**: 2–7.

Pantoja LDM, Moreira-Filho RE, Brito EHS, Aragão TB, Brilhante RSN, Cordeiro RA, Rocha MFG, Monteiro AJ, Quinet YP & Sidrim JJC (2009) Ants (Hymenoptera: Formicidae) as carriers of fungi in hospital environments: an emphasis on the genera *Tapinoma* and *Pheidole*. *J Med Entomol* **46**: 895–899.

Petrovic U, Gunde-Cimerman N & Plemenitas A (2002) Cellular responses to environmental salinity in the halophilic black yeast *Hortaea werneckii*. *Mol Microbiol* **45**: 665–672.

Pfaller MA, Boyken L, Hollis RJ, Messer SA, Tendolkar S & Diekema DJ (2006) *In vitro* susceptibilities of *Candida* spp. To caspofungin: four years of global surveillance. *J Clin Microbiol* **44**: 760–763.

Price MF, Wilkinson ID & Gentry LO (1982) Plate method for detection of phospholipase activity of *Candida albicans*. *Sabouraudia* **22**: 201–207.

Routman E, Miller RD, Phillips-Conroy J & Hartl DL (1985) Antibiotic resistance and population structure in *Escherichia coli* from free-ranging African yellow baboons. *Appl Environ Microb* **50**: 749–754.

Sidrim JJC, Castelo-Branco DSCM, Brilhante RSN, Soares GDP, Cordeiro RA, Monteiro AJ & Rocha MFG (2010) Candida species isolated from the gastrointestinal tract of cockatiels (*Nymphicus hollandicus*): *in vitro* antifungal susceptibility profile and phospholipase activity. *Vet Microbiol* **145**: 324–328.

Solomon GM, Hjelmroos-Koski M, Rotkin-Ellman M & Hammond K (2006) Airborne mold and endotoxin concentrations in New Orleans, Louisiana, after flooding, October through November 2005. *Environ Health Persp* **114**: 1381–1386.

Tawara S, Ikeda F, Maki K *et al.* (2000) *In vitro* activities of a new lipopeptide antifungal agent, FK463, against a variety of clinically important fungi. *Antimicrob Agents Ch* **44**: 57–62.

Tayamen M & Brown JH (1999) A condition index for evaluating larval quality of *Macrobrachium rosenbergii* (De Man, 1879). *Aquac Res* **30**: 917–922.

Valenti WC, Mallasen M & Silva CA (1998) Larvicultura em sistema fechado dinâmico. *Carcinicultura de água doce: Tecnologia para Produção de Camarões, Vol. 1* (Valenti WC, ed), pp. 115–143. FAPESP/IBAMA, S ã o Paulo, Brazil.

Varga VES & Godoy P (2004) *Tinea Nigra*. *Micologia médica à luz de autores contemporâneos, Vol. 1* (Sidrim JJC & Rocha MFG, eds), pp. 124–128. Guanabara Koogan, Rio de Janeiro.

Wegrzyn G & Czyz A (2003) Detection of mutagenic pollution of natural environment using microbiological assays. *J Appl Microbiol* **95**: 1175–1181.

Zeng X, Hou X, Wang Z, Jiang L, Xiong C, Zhou M & Chen Q (2008) Carriage rate and virulence attributes of oral *Candida albicans* isolates from patients with oral lichen planus: a study in an ethnic Chinese cohort. *Mycoses* **52**: 161–165.