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**LEVEDURAS ISOLADAS DE *Macrobrachium amazonicum* E DE
ECOSSISTEMAS AQUÁTICOS: DETECÇÃO DE RESISTÊNCIA A
DERIVADOS AZÓLICOS E O POTENCIAL USO DESSE CAMARÃO
PARA O MONITORAMENTO AMBIENTAL**

**Fortaleza
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Tese apresentada ao Programa de Pós-Graduação em Ciências Veterinárias da Faculdade de Veterinária da Universidade Estadual do Ceará, como requisito parcial para a obtenção do título de Doutor em Ciências Veterinárias.

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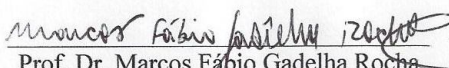
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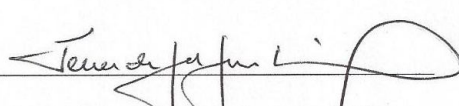
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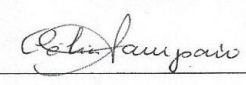
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RESUMO

Em ambientes aquáticos, a crescente poluição causada principalmente pelo despejo de efluentes industriais, sanitários e agrícolas vem se tornando um dos principais problemas ambientais da atualidade. Desta forma, a utilização de bioindicadores se torna uma ferramenta importante para investigação deste tipo de impacto ambiental. Vale destacar que nos últimos anos estudos vem demonstrando que espécies de *Candida* podem ser consideradas como bioindicadores, sendo, muitas vezes, mais isoladas que bactérias indicadoras em ambientes aquáticos poluídos por esgotos. Adicionalmente o uso de sentinelas pode ser eficaz na avaliação de ecossistemas aquáticos poluídos, destacando-se espécies de crustáceos. Assim, buscou-se investigar o uso de *Candida* spp. isoladas do camarão de água doce *Macrobrachium amazonicum* e ecossistemas aquáticos como bioindicadores de poluição ambiental, por meio da análise quali-quantitativa da composição da microbiota e do monitoramento da resistência antifúngica, bem como da avaliação da atividade de bombas de efluxo nas cepas resistentes aos derivados azólicos. Para tanto foram utilizados os tratos gastrintestinais de espécimes adultos de *M. amazonicum* oriundos da lagoa do Catu, Aquiraz, Ceará, Brasil e amostras da água desse ambiente. Os 53 isolados de leveduras, obtidos dos animais, da água de superfície e substrato, foram identificados com base nas características morfológicas e bioquímicas e, em seguida, foram submetidos ao teste de microdiluição em caldo, frente à anfotericina B, itraconazol e fluconazol, segundo a metodologia padronizada pelo CLSI (documento M27-A3). Foi realizada ainda a comparação molecular entre as cepas de *Candida* spp. isoladas do ambiente aquático (n=20) e do camarão (n=7), pelas técnicas M13-PCR fingerprinting e RAPD-PCR. As concentrações inibitórias mínimas (CIM) para anfotericina B, itraconazol e fluconazol foram de 0,03125 a 2 µg/mL, 0,0625 a ≥16 µg/mL e 0,5 a ≥64 µg/mL, respectivamente. De todos os isolados do gênero *Candida*, 20,45% foram resistentes a ambos os derivados azólicos, 25% foram resistentes ao fluconazol e 34,09% foram resistentes ao itraconazol. Vale destacar que dois isolados de *C. guilliermondii* resistentes ao itraconazol também foram resistentes a anfotericina B. Não foi observado resistência aos antifúngicos para os gêneros *Kodamaea*, *Cryptococcus*, *Rhodotorula* e *Trichosporon*. Na avaliação da atividade de bombas de efluxo, foi observado uma redução de CIM das cepas resistentes de oito a 256 vezes em relação a inicial. A análise molecular, através das técnicas M13-PCR fingerprinting e RAPD-PCR, revelou similaridades entre os padrões de bandas entre 11 isolados de *Candida* spp. para

o primer OPQ16 e de oito para o primer M13, indicando forte similaridade entre os isolados. Estes dados apontam a importância da resistência antifúngica como ferramenta bioindicadora de poluição de ambientes de água doce, assim como o uso de *M. amazonicum* como sentinela no monitoramento deste fenômeno.

Palavras-chave: Camarão da Amazônia. *Candida* spp. Resistência antifúngica. Bombas de efluxo. Sentinela ambiental.

ABSTRACT

In aquatic environments, increasing pollution mainly caused by the dumping of industrial, sanitary and agricultural wastewater is becoming one of the main environmental problems nowadays. Therefore, the use of bioindicators becomes an important tool for investigation of this kind of environmental impact. It is noteworthy that over recent years studies have been showing that *Candida* species can be considered a bioindicator, often more isolated than bacteria indicators in polluted aquatic environments by sewage. Additionally the use of sentinels may be effective in the evaluation of polluted aquatic ecosystems, especially species of crustaceans. Thus, it was sought to investigate the use of *Candida* spp. isolated from freshwater prawn *Macrobrachium amazonicum* and aquatic ecosystems as bioindicators of environmental pollution, through qualitative and quantitative analysis of the composition of the microbiota and monitoring of antifungal resistance, well as the evaluation of the activity of efflux pumps in strains resistant to azoles. For that, we used gastrointestinal tracts of adult specimens of *M. amazonicum* originated from Catu Lake, Aquiraz, Brazil and samples of water from this environment. The 53 yeasts isolated, obtained from animals, surface water and substrate, were identified based on morphological and biochemical characteristics, and then were submitted to the broth microdilution test, against to amphotericin B, itraconazole and fluconazole, according to methodology standardized by CLSI (document M27-A3). It was performed a molecular comparison of strains of *Candida* spp. isolated from the aquatic environment (n=20) and prawns (n=7), by the M13-PCR fingerprinting and RAPD-PCR techniques. The minimal inhibitory concentration (MIC) to amphotericin B, itraconazole and fluconazole were 0.03125 to 2 µg/mL, 0.0625 to ≥16 µg/mL and 0.5 to ≥64 µg/mL, respectively. Of all isolates of the *Candida* genus, 20.45% were resistant to both azole derivatives, 25% were resistant to fluconazole and 34.09% were resistant to itraconazole. It is noteworthy that two isolates of *C. guilliermondii* resistant to itraconazole were also resistant to amphotericin B. Was not observed resistance to antifungals for genera *Kodamaea*, *Cryptococcus*, *Rhodotorula* and *Trichosporon*. In evaluating the activity of efflux pumps, a reduction of MIC of resistant strains from eight to 256 times compared to initial was observed. Molecular analysis through the techniques M13-PCR fingerprinting and RAPD-PCR revealed similarities among the band patterns from 11 isolates of *Candida* spp. for the primer OPQ16 and eight for the primer M13, indicating strong similarity among the isolates. These findings indicate the importance of antifungal resistance as a

bioindicator tool of the freshwater environments pollution, as well as the use of *M. amazonicum* as a sentinel monitoring of this phenomenon.

Palavras-chave: Amazon prawn. *Candida* spp. Antifungal resistance. Efflux pumps. Environmental Sentinel.

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LISTA DE ABREVIATURAS E SIGLAS

ATCC – American Type Culture Collection
CEMM – Centro Especializado em Micologia Médica
CFU – Colony Forming Units (Unidades Formadoras de Colônia)
CIM – Concentração inibitória mínima
CLSI – Clinical Laboratory Standards Institute
HAP – Hidrocarbonetos aromáticos policíclicos
LACAR – Laboratório de Carcinicultura da Universidade Estadual do Ceará
MDR – Multidroga Resistente
MFS – *Major Facilitators*
MIC - Minimum Inhibitory Concentrations
NBD – *Nucleotide Binding Domain*
PCR-REA - *Polymerase Chain Reaction-Restriction Endonuclease Analysis*
P-gp – Glicoproteína P
RAPD-PCR – *Random Amplified Polymorphic DNA- Polymerase Chain Reaction*
S – *Sediment*
SISBIO – Sistema de Informação e Autorização em Biodiversidade
SW – *Surface Water*
UPGMA – *Unweighted Pair Group Method*

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1 INTRODUÇÃO

Na literatura científica, há registros relatando a importância de leveduras para a aquicultura, tanto como componentes da microbiota, como causadores de enfermidades em variados grupos de interesse aquícola (LEAÑO et al., 2005; GATESOUBE, 2007). Nesses registros, distintas espécies de leveduras foram isoladas de uma grande variedade de ecossistemas aquáticos, mas apenas um número limitado é prevalente, com destaque para as espécies dos gêneros *Candida*, *Cryptococcus*, *Rhodotorula*, *Saccharomyces* e *Trichosporon* (ROSA et al., 1995). Adicionalmente, *Candida* spp. representam o maior número de espécies isoladas em cultivos de camarão (JOHNSON; BUENO, 2000).

Nos últimos anos, alguns estudos demonstraram a alta frequência de isolados de *Candida* spp. resistentes à antifúngicos, oriundos de ambientes aquáticos e embora associem essa manifestação com o risco à saúde pública, não se sabe ao certo qual a causa deste fenômeno (MEDEIROS et al., 2008; BRANDÃO et al., 2010). Em pesquisa realizada com leveduras isoladas de camarões da espécie *Macrobrachium amazonicum*, oriundos da lagoa do Catu, Ceará, Brasil, foram obtidos 28% de isolados de *Candida* spp. com resistência *in vitro* a derivados azólicos (BRILHANTE et al., 2010). Tal observação despertou interesse pela busca das possíveis causas da ocorrência de cepas resistentes de *Candida* spp., no ambiente natural.

Embora existam muitos trabalhos abordando o monitoramento do perfil de sensibilidade de leveduras isoladas de amostras clínicas humanas (PFALLER et al., 2010), ainda são escassos os trabalhos com leveduras isoladas do ambiente. Demonstrando, assim, a importância da realização de estudos para a melhor compreensão do fenômeno de resistência em amostras oriundas do ambiente aquático.

Sabe-se que, de modo em geral, existem dois mecanismos primordiais para o desenvolvimento de resistência a derivados azólicos: o primeiro consiste no desenvolvimento de bombas de efluxo ativo; e o segundo na alteração da enzima lanosterol C14 α -desmetilase, molécula-alvo dos derivados azólicos (KANAFANI; PERFECT, 2008; MANASTIR et al., 2009).

Nos estudos realizados por Brilhante et al. (2011), analisando a microbiota gastrointestinal, por leveduras, em camarões da espécie *M. amazonicum*, observou-se o fenômeno de resistência ao itraconazol e ao fluconazol, em isolados de *Candida* spp. Acredita-se que a observação desse fenômeno esteja associada à presença de agentes químicos potencialmente mutagênicos, passíveis de serem encontrados nos efluentes

industriais que contaminam ambientes aquáticos. Adicionalmente, a superexpressão de bombas de efluxo pode estar relacionada com sua atividade inespecífica, que esta diretamente ligada com a desintoxicação celular e sobrevivência dessas leveduras no ambiente aquático poluído.

Assim, se torna necessário a realização de estudos avaliando o perfil de sensibilidade a antifúngicos em leveduras oriundas de ambientes aquáticos para a melhor compreensão deste fenômeno em ambientes com diferentes níveis de degradação ambiental.

2 REVISÃO DE LITERATURA

2.1 Camarão da Amazônia (*Macrobrachium amazonicum*)

A expressão “camarão de água doce” é utilizada para caracterizar tanto espécies que têm todo seu ciclo de vida restrito a esse ambiente, como espécies que necessitam de água salobra no início de seu desenvolvimento e de água doce depois da metamorfose (COELHO; RAMOS-PORTO; SOARES, 1981). Embora sejam também chamados de camarões, como os de água salgada, os de água doce são evolutivamente mais próximos das lagostas, expressando muitas semelhanças com estas, principalmente quanto aos hábitos reprodutivos, pois as fêmeas de ambas as espécies incubam seus ovos no abdômen até a eclosão das larvas (ISMAEL; NEW, 2000).

Os camarões de água doce são crustáceos decápodes pertencentes à subordem Pleocyemata e família Palaemonidae (HOLTHUIS; NG, 2010). O gênero mais representativo desta família é o *Macrobrachium*, que segundo Holthuis e Ng (2010), é circuntropical e nativo em todos os continentes, exceto na Europa e Antártida. Atualmente, existem em todo o mundo cerca de 243 espécies de camarões pertencentes a esse gênero (DE GRAVE; FRANSEN, 2011), dentre as quais 46 são registradas nas Américas e 19 no Brasil (MELO, 2003; SANTOS; HAYD; ANGER, 2013). Vale destacar que, a maioria das espécies de camarão de água doce de interesse comercial pertence ao gênero *Macrobrachium* (MELO, 2003).

M. amazonicum pertence ao grupo de espécies continentais de desenvolvimento larval completo, e ocorre desde a bacia do Orinoco, passando pelo rio Amazonas, até a bacia do rio Paraguai (Figura 1) (HOLTHUIS, 1952).



(Fonte: PAIVA, 2014a)

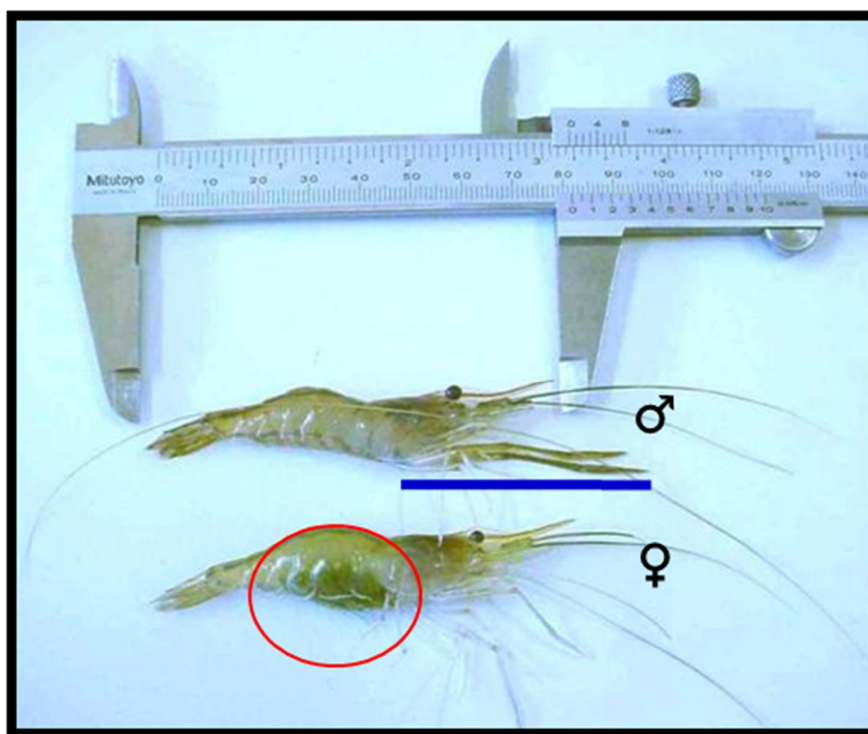
Figura 1 – Distribuição geográfica de *Macrobrachium amazonicum*.

Decápode de água doce de maior importância econômica no Sudeste do Continente Sul-americano, *M. amazonicum* possui elevado potencial para a aquicultura, sendo consumido por povos indígenas e brasileiros de todos os grupos econômicos, e amplamente explorada pela pesca artesanal no Norte e Nordeste do Brasil, chegando a representar 85% do pescado de camarão selvagem de água doce no Brasil, no final da década de 1990 (MACIEL; VALENTI, 2009).

Os espécimes apresentam rostro longo e delgado com margem superior provida de nove a 12 dentes, margem inferior com oito a dez dentes distribuídos irregularmente; carapaça e abdômen lisos e transparentes e télson terminando em uma extremidade aguda com dois pares de espinhos na margem posterior. A segunda pata no adulto é a mais forte. Machos adultos exibem mero, carpo e própode cobertos por espínulos curtos os quais estão ausentes nas fêmeas (MELO, 2003). O sexo é separado, além de indicar

características morfológicas externas que permitem distinguir facilmente, em exemplares maduros, machos e fêmeas (MORAES-VALENTI; VALENTI, 2010).

Machos e fêmeas de camarões palaemonídeos apresentam compleição física semelhante até atingirem a maturidade sexual, quando têm início os processos reprodutivos. Desde então, as fêmeas destinam suas reservas energéticas à produção e à incubação dos ovos, enquanto os machos direcionam o gasto energético ao crescimento somático, fazendo com que se tornem os maiores indivíduos da população (AMMAR; MÜLLER; NAZARI, 2001) (Figura 2).



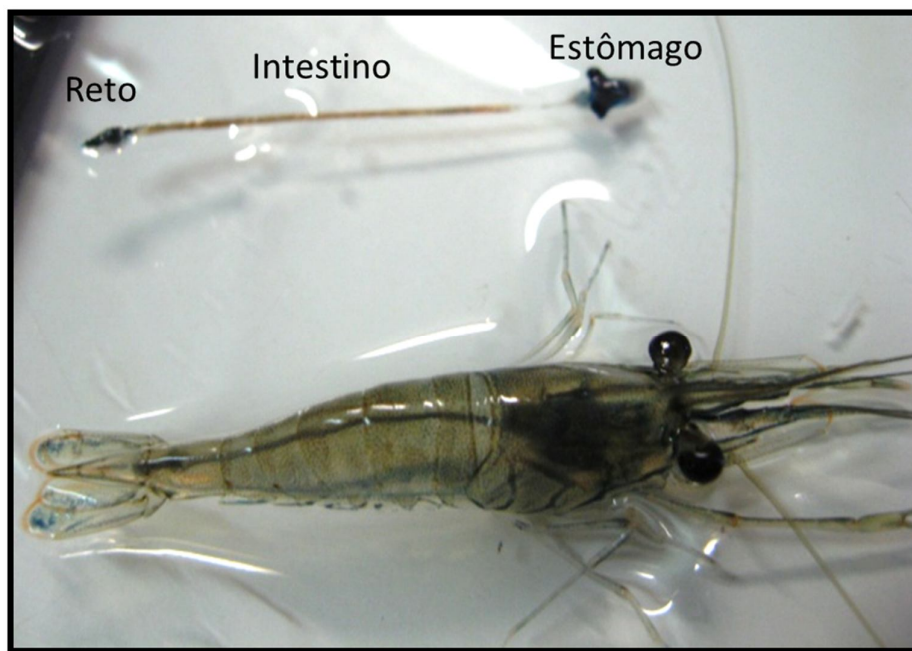
(Fonte: PAIVA, 2005)

Círculo vermelho = Ovos na câmara de incubação. Reta azul = Segundo par de pereiópodo mais desenvolvido no macho.

Figura 2 – Macho e fêmea de *M. amazonicum*, morfologia externa.

Os juvenis são translúcidos, com o segundo par de pereiópodes delgado. As fêmeas de *M. amazonicum* normalmente são menores do que os machos. Os ovos se desenvolvem em uma câmara de incubação, formada pelo arqueamento e alongamento da pleura abdominal. Estes são geralmente pequenos e passam, durante o desenvolvimento embrionário, da tonalidade verde-escuro, no início, a verde-claro, até apresentarem uma coloração verde-acinzentada, cinza-clara ou esbranquiçada quando se encontram próximo à eclosão das larvas (MORAIS-VALENTI; VALENTI, 2010).

O sistema digestório em camarões do gênero *Macrobrachium* é completo e tubular; inicia-se com a boca anteroventral, percorre dorsalmente o corpo do animal e termina em um ânus localizado na base do télson (Figura 3). Segundo Ismael e New (2000), o sistema digestório se divide em três regiões - anterior, média e posterior - e inclui também uma glândula digestiva, cecos pilóricos e divertículos. O intestino anterior está situado na porção dorsal do cefalotórax e possui esôfago e estômago, este com duas câmaras - a cardíaca e a pilórica - responsáveis pelos mecanismos de trituração e filtração e que, junto com as enzimas iniciam o processo digestivo. O moinho gástrico, um mecanismo de trituração formado por ossículos na câmara anterior do intestino anterior, está presente em todos os decápodes, exceto em alguns carídeos, como os camarões de água doce do gênero *Macrobrachium*. Desta maneira, a mastigação nestes animais depende exclusivamente das mandíbulas que quebram e separam as partículas alimentares, encaminhando-as para o intestino médio.



(Fonte:PAIVA, 2010)

Figura 3 – Sistema digestório de *M. amazonicum*.

2.2 Leveduras na Aquicultura

De acordo com De Hoog et al. (2000), levedura é um termo descritivo para qualquer fungo que se reproduz por brotamento, cuja unidade funcional é o blastoconídio. Os fungos denominados de leveduras se encontram classificados nas classes *Hemiascomycetes*, da divisão *Ascomycota*, e *Hymenomycetes* e *Urediniomycetes*, da divisão *Basidiomycota*.

Artificialmente, as leveduras são definidas como organismos unicelulares, brancos ou avermelhados. A formação de cadeias celulares coesas (pseudomicélio) é comum, inclusive, algumas espécies produzem hifas verdadeiras e artroconídios. Estes fungos são tratados como leveduras por serem filogeneticamente semelhantes a uma das classes retrocitadas (DE HOOG et al., 2000).

A maioria das leveduras clinicamente relevantes se reproduz por processos vegetativos, cujos principais mecanismos são o brotamento celular, a fissão celular e a formação de artroconídios (DE HOOG et al., 2000).

Trabalhos avaliando o papel de leveduras em espécies de interesse para aquicultura são escassos e relatam, principalmente, o papel como patógeno e como representante da microbiota de peixes e crustáceos de interesse comercial. Nos estudos realizados com esses animais, os principais gêneros de leveduras isolados são *Rhodotorula*, *Saccharomyces*, *Trichosporon*, *Candida* e *Cryptococcus* (BRUCE; MORRIS, 1973; LEAÑO et al., 2005; MENDONÇA-HAGLER; HAGLER, 1989; PAGNOCCA).

Brilhante et al. (2011) realizaram um estudo em um lago localizado no Nordeste do Brasil (Ceará), com a utilização de camarões para o isolamento de leveduras desse ambiente, e observaram cepas de *Candida* spp. resistentes a derivados azólicos. Nesse estudo, os autores sugeriram a utilização deste crustáceo como um sentinela ambiental e as leveduras como bioindicadores para presença de poluentes, por meio da avaliação do perfil de sensibilidade a antifúngicos *in vitro*.

2.3 Sentinelas para o isolamento de leveduras

A microbiota intestinal de invertebrados aquáticos é semelhante a do meio ambiente em que estão inseridos, assim leveduras podem ser mais facilmente isoladas com origem nestes animais do que diretamente do ambiente aquático (HAGLER et al.,

1995). Desta forma, estes animais, especialmente moluscos bivalves e camarões, podem ser utilizados para melhorar o isolamento de leveduras em ambientes aquáticos, que, por sua vez, atuam como bioindicadores para a análise da saúde ambiental (HAGLER et al., 1995; KUTTY; PHILIP, 2008).

O termo sentinela foi utilizado pela primeira vez na década de 1950 para descrever os bivalves utilizados a fim de detectar e mapear radioatividade (WASHINGTON, 1984) e, desde então, o programa *Mussel Watch* é utilizado em vários estudos que envolvem a utilização de bioacumuladores (BEEBY, 2001). Em geral, sentinelas são monitores biológicos capazes de acumular poluentes sem receber efeitos secundários significativos e são, principalmente, utilizados para medir a quantidade de um determinado poluente, com vistas a aumentar a sensibilidade de determinada técnica analítica e/ou para facilitar a interpretação de um sinal complexo de poluição. Com efeito, as espécies sentinelas podem ser classificadas em três grandes grupos: 1) espécies monitoras, cujas funções biológicas são reduzidas na presença de certos poluentes; 2) espécies indicadoras, apontando a presença de um desequilíbrio por aumento ou diminuição de sua ocorrência no ambiente; e 3) espécies sentinelas, que acumulam poluentes em seus tecidos sem sofrer danos significativos, permitindo a quantificação da fração biodisponível de uma determinada substância química em um ecossistema. Adicionalmente, os sentinelas também podem ser descritos como organismos que podem acumular algumas espécies de microrganismos bioindicadores nos seus sistemas (BEEBY, 2001).

Variados organismos são sugeridos como sentinelas, tais como anelídeos (BARUS; JARKOVSKY; PROKES, 2007), peixes (ZORITA et al., 2008), moluscos (BEEBY, 2001; MAZZIA et al., 2011), abelhas (LAMBERT et al., 2012), macroinvertebrados bentônicos (BRILHANTE et al., 2011; CHIBA; PASSERINI; TUNDISI, 2011), anfíbios (HOPKINS, 2007), aves (BRILHANTE et al., 2012) e mamíferos (MARIANO et al., 2009). Entre essas espécies, macroinvertebrados bentônicos são particularmente interessantes em razão de suas características como filtradores e da facilidade de recuperação desses animais em ambientes aquáticos (BEEBY, 2001; MAZZIA et al., 2011). Os estudos com estes animais têm abordado o acúmulo de metais pesados, hidrocarbonetos, pesticidas e outros poluentes, em seus tecidos (MAHMOUD; TALEB, 2013).

Em 1993, o programa internacional *Mussel Watch* inseriu o Brasil em suas análises para o monitoramento de contaminantes químicos costeiros utilizando bivalves, e foi demonstrada a necessidade de avaliar melhor a extensão e a gravidade das

concentrações de hidrocarbonetos aromáticos policíclicos (HAP) nas zonas costeiras do país e uma avaliação dos efeitos adversos em áreas onde HAP têm elevados concentrações (FARRINGTON; TRIPP, 1994) (Figura 4).



(Fonte: FARRINGTON; TRIPP, 1994)

Figura 4 – Programa internacional *Mussel Watch*.

Considerando-se que estes compostos químicos podem causar alterações na expressão ou na sequência de genes específicos (KEENAN et al., 2007; MULLER et al., 2007), que, por sua vez, podem conduzir a resistência a azólicos (KANAFANI; PERFECT, 2008), parece plausível pensar que o acúmulo de compostos químicos nos tecidos pode aumentar a taxa de resistência a azólicos entre leveduras na microbiota de animais sentinelas.

Assim, Brilhante et al. (2012), por exemplo, avaliaram a sensibilidade de *Candida* spp. isoladas do trato gastrointestinal de aves de rapina e observaram elevada proporção de cepas resistentes a azólicos, demonstrando o potencial papel destas aves como sentinelas a fim de isolar *Candida* spp. para o monitoramento ambiental. Quanto aos ambientes aquáticos, Andrade et al. (2010), isolaram de *M. amazonicum*, coletados do

ambiente natural, diversas espécies de bactérias resistentes e sugeriram que essa resistência estava associada ao emprego inadequado de antibacterianos em fazendas de carcinicultura e piscicultura.

Da mesma forma, Brilhante et al. (2011) sugeriram que o *M. amazonicum* seria um bom sentinela para monitorar a resistência a antifúngicos entre *Candida* spp. ambientais, enquanto que estas *Candida* spp. resistentes poderiam ser um indicador importante para a presença de compostos químicos em um determinado ambiente aquático.

2.4 Leveduras e relação com ambientes aquáticos alterados

Muitos ensaios têm demonstrado uma tendência mundial no uso de leveduras oriundas do ambiente e de animais como uma ferramenta para avaliar a qualidade dos ambientes aquáticos (CHEN; YANAGIDA; CHEN, 2009; COELHO et al., 2010; HAGLER; MENDONÇA-HAGLER, 1981; SIMARD, 1971). As leveduras mais comumente isoladas nesses estudos pertencem aos gêneros *Candida*, *Cryptococcus*, *Rhodotorula*, *Saccharomyces* e *Trichosporon* (HAGLER et al., 1995), com destaque para as espécies *C. famata*, *R. mucilaginosa* e *C. laurentii*, que têm sido considerados bioindicadoras de poluição em ambientes aquáticos (ARVANITIDOU et al., 2002; BRILHANTE et al., 2011; CHUN, 1984; COELHO et al., 2010; HAGLER et al., 1995; HAGLER; MENDONÇA-HAGLER, 1981; LIBKIND et al., 2003; MEDEIROS et al., 2008; SLÁVIKOVÁ; VADKERTIOVÁ; KOCKOVÁ-KRATOCHVÍLOVÁ, 1992).

Vários autores asseguram que a análise da microbiota leveduriforme é um instrumento válido para a avaliação do estado de eutrofização em um determinado ambiente aquático (ARVANITIDOU et al., 2002; BRILHANTE et al., 2011; MEDEIROS et al., 2008; ROSA et al., 1990). A utilização de leveduras como bioindicadores foi inicialmente proposto na década de 1970, baseado num estudo realizado no rio St. Lawrence, no Canadá. Esse estudo demonstrou que em áreas localizadas perto de esgoto, muitas espécies de leveduras cresceram abundantemente e o número de isolados e a frequência de recuperação poderiam ser usados como parâmetros para avaliar a poluição (SIMARD, 1971). Assim, Rosa et al. (1990) investigaram a ocorrência de leveduras e coliformes fecais em cinco estações de coleta de água na lagoa Olhos D'Água, no Estado de Minas Gerais, Brasil, e observaram que, em três estações, o

número de leveduras recuperadas foi maior do que a de coliformes fecais, fato sugestivo de que esses fungos poderiam ser melhores bioindicadoras de poluição em fontes de água.

Nas pesquisas realizadas em regiões de clima temperado, como Argentina (LIBKIND et al., 2003) e Austrália (LATEGAN et al., 2012), leveduras carotenogênicas, como *Rhodotorula* spp., foram importantes para a avaliação da influência antrópica no ambiente aquático, considerando que estas leveduras só foram isoladas em áreas degradadas pela atividade antrópica. Além disso, *Rhodotorula* spp. podem ser usadas para monitorar a qualidade de águas subterrâneas, como mostrado por Lategan et al. (2012), que avaliaram a diversidade de fungos em fontes de águas subterrâneas superficiais na Austrália.

Adicionalmente, leveduras podem ser utilizadas como bioindicadores para avaliar as alterações ambientais, por meio de dois mecanismos: o primeiro, correlaciona a taxa de recuperação de várias espécies de leveduras para as distintas condições ambientais, tendo em conta a presença das fontes de poluição, como esgotos e efluentes industriais; o segundo avalia a presença de alterações fenotípicas nestes microrganismos recuperados em ambientes alterados. Esses mecanismos fundamentam-se na utilização de leveduras como bioindicadores de alterações nos ambientes aquáticos (BRILHANTE et al., 2011; HAGLER et al., 1995; HAGLER; MENDONÇA-HAGLER 1981; MEDEIROS et al., 2008; ROSA et al., 1990; SIMARD, 1971).

2.5 *Candida* spp. como bioindicadores da qualidade do ecossistema aquático

As pesquisas envolvendo o isolamento de leveduras demonstram a onipresença do gênero *Candida*. Este gênero mostra ser predominante, quando se considera o número elevado de amostras, bem como a elevada taxa de recuperação, independentemente da estação do ano (RASPOR; ZUPAN, 2006). Este fato se torna ainda mais evidente quando as amostras são de fontes aquáticas, ademais, pesquisas propõem o uso de *Candida* spp. como bioindicadores ambientais (COELHO et al., 2010; HAGLER et al., 1995; MEDEIROS et al., 2008). Em pesquisas que visam correlacionar a degradação ambiental às atividades antrópicas, tais como esgotos domésticos e efluentes industriais e agrícolas, *Candida* spp. são espécies de interesse, uma vez que são comumente associadas com a presença de fezes humanas e de animais (HAGLER, 2006; HAGLER; MENDONÇA-HAGLER, 1981).

C. famata é a espécie mais comumente isolada de fontes aquáticas e seu isolamento com taxas elevadas caracteriza a degradação ambiental com esgotos domésticos (BRANDÃO et al., 2010; BRILHANTE et al., 2011; MEDEIROS et al., 2008). Além disso, essa espécie tem associação com o isolamento de coliformes fecais, o que demonstra o seu papel como um indicador de poluição em ambientes aquáticos (MEDEIROS et al., 2008; ROSA et al., 1990). A predominância de *C. famata* nesses ambientes pode estar relacionada com a sua grande capacidade de resistir a baixas temperaturas, variadas salinidades e variações de osmolaridade (RASPOR; ZUPAN, 2006). Ademais, além de indicar a eutrofização, esta espécie parece estar intimamente associada com a remoção de poluentes, como os hidrocarbonetos (HAP), com origem na água contaminada (FARAG; SOLIMAN, 2011).

C. guilliermondii também foi isolada em ambientes aquáticos, especialmente aqueles eutrofizados ou contaminados com esgotos domésticos e efluentes industriais, como demonstrado por Medeiros et al. (2008), Brandão et al. (2010) e Brilhante et al. (2011). Papon et al. (2013) sugeriram que essa espécie é capaz de usar várias fontes de carbono, incluindo os hidrocarbonetos, contribuindo para a biorremediação de solos e água contaminados por derivados de petróleo.

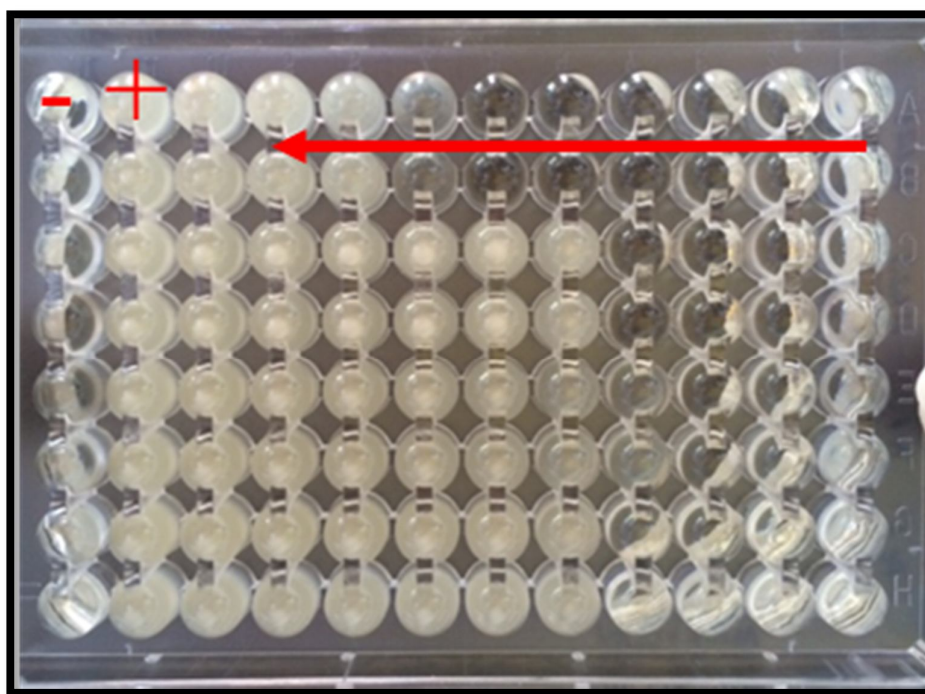
Medeiros et al. (2008), ao avaliarem a diversidade de leveduras em lagos e rios da região Sudeste do Brasil, descobriram que o gênero *Candida* foi o que exibiu o maior número de espécies e o maior quantitativo de isolados, dos quais 50% foram resistentes ao itraconazol. Assim, com base nestes resultados, os autores sugeriram que, nos ambientes aquáticos investigados, a presença de *Candida* spp. resistentes a derivados azólicos estava associada com as atividades antrópicas a que estes ambientes foram submetidos.

2.6 Teste de sensibilidade a antifúngicos

O conhecimento do perfil de sensibilidade a antifúngicos de determinada região é de grande importância para o estabelecimento de condutas terapêuticas e profiláticas adequadas. Em 1985, o Comitê da Área de Micologia do Clinical Laboratory Standards Institute (CLSI) publicou o seu primeiro relatório, no qual se delinearam os resultados de um pequeno estudo colaborativo. Constatou-se que 20% dos laboratórios membros da instituição realizavam testes de sensibilidade a agentes antifúngicos, na maioria das vezes, com *Candida* spp., empregando o método de diluição em caldo, e obtinham

resultados de concentração inibitória mínima (CIM) discrepantes. Desde então, foi decidido desenvolver e padronizar uma metodologia reprodutível e exequível para laboratórios de rotina. Em 1997, a Norma M27-A foi publicada, especificando os pontos de corte para os antifúngicos disponíveis. Em 2002, a Norma M27-A2 padronizou as faixas de referência de CIM 24 e 48 horas, e em 2008 a Norma M27-A3 revisou esses dados para drogas previamente estabelecidas e recém-lançadas (CLSI, 2008).

O teste de microdiluição em caldo é realizado em placas acrílicas estéreis, com 96 poços em formato de U, e consiste na exposição de um inóculo definido de um determinado microorganismo a concentrações conhecidas das drogas testadas, sendo possível observar o efeito destas sobre o crescimento fúngico. A leitura final determina a menor concentração da droga, capaz de inibir o crescimento do microorganismo, denominada de concentração inibitória mínima (CIM) (CLSI, 2008) (Figura 5).



Controle negativo = Avaliação da esterilidade do meio e da droga. Controle positivo = Avaliação do crescimento e esterilidade do inóculo. Sentido da diluição.

(Fonte: PAIVA, 2014b)

Figura 5 – Microdiluição em placas de 96 poços em formato de U para *Candida* spp.

O objetivo final dos testes de sensibilidade é prever a resposta dos pacientes à terapia a ser instituída. Muitos fatores, no entanto, além do perfil de sensibilidade *in vitro*, influenciam a resposta clínica, como o sítio de infecção, o *status* imunológico do hospedeiro, a farmacocinética da droga e a adesão do paciente à terapia. Portanto, o estabelecimento da correlação clínica direta entre os valores de CIM e o desfecho terapêutico ainda é limitado na terapia antifúngica (REX; PFALLER, 2002; HOSPENTHAL; MURRAY; RINALDI, 2004). A realização de testes de sensibilidade, contudo, se torna importante, uma vez que pode orientar a instituição da terapia antifúngica mais adequada, direcionando o paciente aos 90% de sucesso terapêutico (REX; PFALLER, 2002).

Em geral, a taxa de resistência aos azólicos permanece baixa, entre a maioria das espécies de *Candida* spp., variando de 1-2,1% em *C. albicans*, de 0,4-4,6% em *C. parapsilosis* de 1,4-6,6% em *C. tropicalis*. No entanto, *C. glabrata*, a segunda espécie mais prevalente em infecções fúngicas sistêmicas nos Estados Unidos, exprime crescente resistência ao fluconazol, cuja taxa de resistência aumentou de 7 para 12%, de 2001 a 2004 (KANAFANI; PERFECT, 2008).

Existem muitos trabalhos com o monitoramento do perfil de sensibilidade a drogas de leveduras isoladas de amostras clínicas humanas, como pode ser observado nas metanálises realizadas por Pfaller e Diekema (2007) e Pfaller et al. (2010). São escassos, no entanto, os trabalhos com leveduras isoladas de animais. Brito et al. (2009) observaram resistência intrínseca a cetoconazol, fluconazol e itraconazol em isolados de *C. albicans* e de *C. tropicalis* oriundos de cães, e Brilhante et al. (2010), em um estudo que foi realizado com calopsitas (*Nymphicus hollandicus*), ave pertencente à família *Psittacidae*, observaram fenômeno de resistência a itraconazol e fluconazol, em isolados de *C. albicans*.

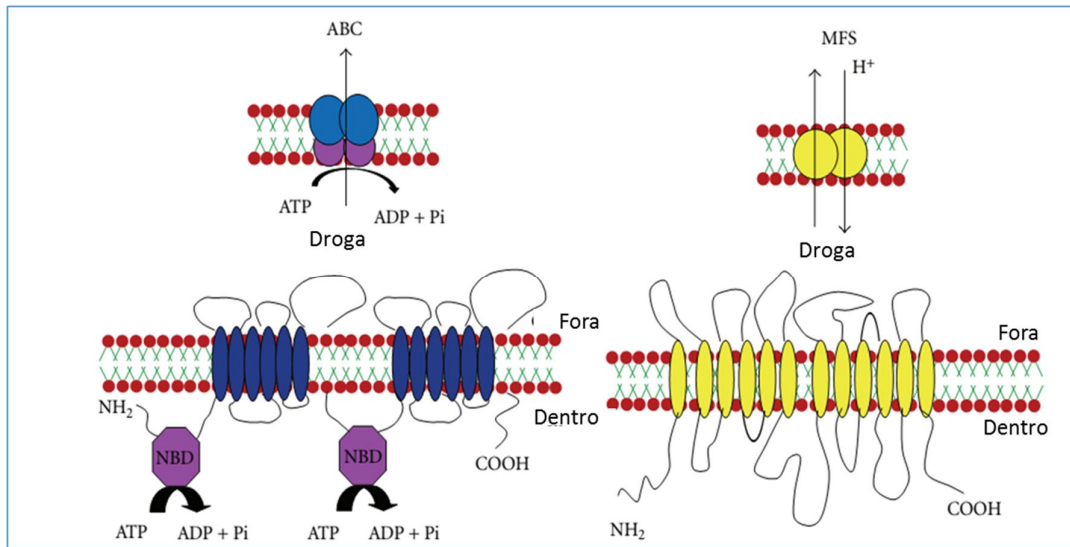
Ante o exposto, diversas espécies de leveduras isoladas de ambientes aquáticos e de animais de interesse aquícola são conhecidas como patógenos e podem comprometer a saúde humana (PFALLER; DIEKEMA, 2007). Algumas cepas de leveduras, previamente consideradas colonizadores simples ou contaminantes, são resistentes a diversas drogas antifúngicas disponíveis e podem causar infecções em humanos. Estas infecções são descritas com frequência crescente em relação às permissivas condições ambientais, a pressão seletiva antifúngica e a pacientes imunodeprimidos (CANUTO; RODERO, 2002; ENOCH; LUDLAM; BROWN, 2006; PFALLER; DIEKEMA, 2007).

Medeiros et al. (2008) isolaram leveduras de amostras de água e de sedimento de lagos poluídos, localizados no Sudeste do Brasil, e traçaram o perfil de sensibilidade a varias drogas antifúngicas. De todos os isolados submetidos ao teste de sensibilidade *in vitro* (68 leveduras), 13% foram sensíveis ao cetoconazol, 79% para fluconazol, 50% para itraconazol, 31% para terbinafina e 78% das cepas foram sensíveis a anfotericina B. Sete isolados de diferentes espécies de *Candida* foram resistentes a todos esses antifúngicos.

Brilhante et al. (2011) observaram que 28,6% (4/14) do total de isolados de *Candida* spp. do trato digestório de *M. amazonicum* coletados no ambiente natural foram resistentes a esses derivados azólicos. Este fenômeno de resistência aos derivados azólicos despertou a curiosidade de buscar as possíveis causas associadas a este fenômeno no ambiente.

2.7 Resistência antifúngica a derivados azólicos como estratégia para o monitoramento ambiental

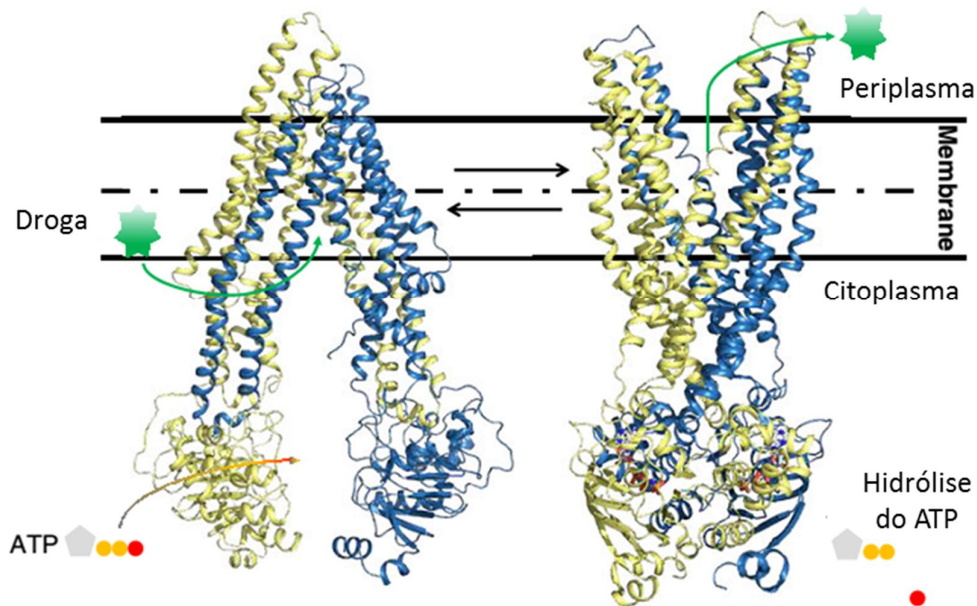
De maneira em geral, existem dois tipos principais de mecanismos envolvidos no desenvolvimento de resistência aos derivados de azólicos. O primeiro mecanismo está associado com o desenvolvimento de bombas de efluxo ativo, codificadas pelos genes *CDR1* e *CDR2*, que pertencem à superfamília *ATP binding cassette*, e o gene *MDR1*, que pertence à classe *Major Facilitators*. A superexpressão destes genes evita o aumento da concentração do fármaco no interior da célula, consequentemente, diminuindo a sua eficácia. O aumento da regulação de *CDR1* e *CDR2* leva à resistência a quase todos os antifúngicos, enquanto que aumento da regulação do *MDR1* conduz à resistência ao fluconazol (KANAFANI; PERFECT, 2008) (Figuras 6 e 7). O segundo mecanismo de resistência está associado a alterações na enzima lanosterol C14 α -desmetilase, molécula-alvo dos derivados de azólicos, que é codificada pelo gene *ERG11* (Figura 8). Superexpressão ou mutações nestes genes diminui a sensibilidade ou leva a resistência aos derivados azólicos (KANAFANI; PERFECT, 2008; MANASTIR; ERGON; YÜCESOY, 2009).



O NBDs (Nucleotide Binding Domains) das bombas ABC são responsáveis pela hidrólise de ATP, o que facilita a retirada da droga do meio intracelular, enquanto os transportadores MFS utilizam gradiente de prótons para expelir drogas.

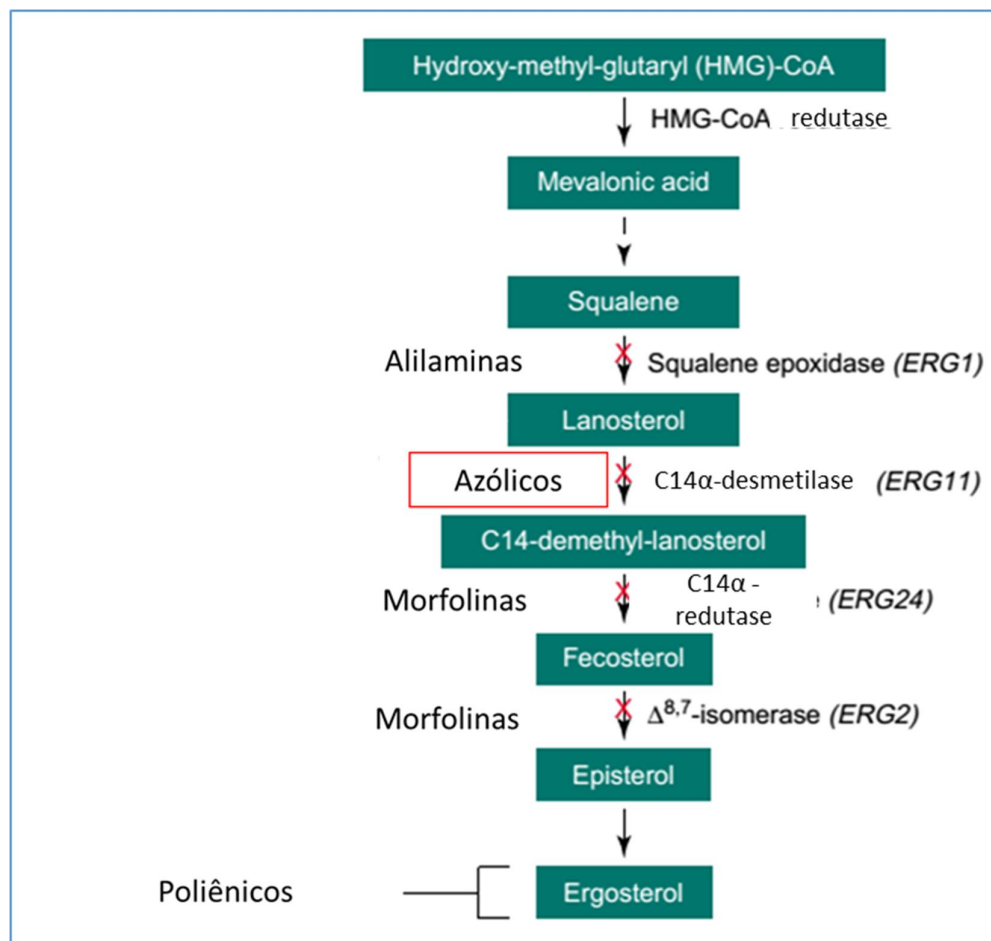
(Fonte: PRASAD; SHARMA; RAWAL, 2011)

Figura 6 – Representação das bombas de efluxo ABC *binding cassette* e transportadores MFS (Major Facilitators) de *Candida* spp.



(Fonte: MARTINEZ; FALSON, 2014)

Figura 7 – Representação da estrutura das bombas de efluxo ABC *binding cassette*.



(Fonte: LUPETTI et al., 2002)

Figura 8 - Mecanismo de ação de drogas antifúngicas afetando a via biossintética do ergosterol.

Adicionalmente, o desenvolvimento da resistência aos azólicos entre *Candida* spp. pode estar relacionado com as atividades antrópicas, uma vez que a resistência antifúngica é mediada principalmente por mudanças na expressão gênica e/ou sequência de nucleotídeos do gene (FENG et al., 2010) e resíduos industriais e poluentes são capazes de promover tais alterações (KEENAN et al., 2007; MÜLLER et al., 2007). Foi demonstrado que os microrganismos resistem aos efeitos tóxicos de variados tipos de solventes, derivados de petróleo e agentes mutagênicos, mediante a ação destas bombas de efluxo, que exportam para o ambiente extracelular vários substratos, como metais pesados e seus derivados químicos, incluindo cádmio, arsenato, hexano, tolueno, xileno, e outros (BRUINS; KAPIL; OEHME, 2000; KIEBOOM et al., 1998). Além disso, bombas de efluxo também são responsáveis por diversos fenômenos biológicos, atuando

em processos celulares vitais, como a secreção de peptídeos envolvidos na comunicação celular, os feromônios, regulação mitocondrial, adaptação ao estresse e desintoxicação (JUNGWIRTH; KUCHLER, 2006).

Efetivamente, a resistência aos derivados azólicos mediada pelo aumento da atividade de bombas de efluxo ativo pode ser avaliada *in vitro* com base em substâncias moduladoras que diminuem a atividade das bombas de efluxo, dentre as quais se destacam as drogas neurolépticas/antidopaminérgicas, como as fenotiazinas (KOLACZKOWSKI; MICHALAK; MOTOHASHI, 2003) e os derivados de butirofenona (RAMÓN-GARCÍA et al., 2011).

As fenotiazinas e as butirofenonas são similares, antagonistas de receptores dopaminérgicos, ligando-se aos receptores de histamina H1 e dopamina D2, respectivamente, agindo como agentes anti-histamínicos ou neurolépticos para o controle de transtornos psicóticos (RAMÓN-GARCÍA et al., 2011; SMITH; COX; SMITH, 2012). As fenotiazinas apresentam atividade contra bombas de efluxo ativo que pertencem à superfamília *ATP binding cassette* (ABC) e à classe *Major Facilitator* (MFS), enquanto as butirofenonas apresentam atividade contra bombas MFS (TEGOS et al., 2011).

Diferentes estudos demonstraram que essas drogas expressam atividade antibacteriana (OHLOW; MOOSMANN, 2011; RAMÓN-GARCÍA et al., 2011; TEGOS et al., 2011), bem como foi demonstrada atividade de fenotiazina contra *Candida* spp. (CASTELO-BRANCO et al., 2013; GALGÓCZY et al. 2011) e sobre a atividade das proteínas de bombas de efluxo do fungo *Saccharomyces cerevisiae*. Por meio da adição de concentrações subinibitórias, foi possível reverter a resistência a cetoconazol em uma cepa multidroga resistente (MDR), em decorrência da produção elevada de Pdr5p, homólogo da proteínas Cdr1p e Cdr2p que pertencem à superfamília de bombas de efluxo ABC. Ademais, o efeito sinérgico das fenotiazinas combinadas com cetoconazol contra a superprodução da proteína Pdr5p em cepas MDR se assemelharam notavelmente ao efeito da deleção do gene *PDR5*, que codifica esta proteína, o que destaca a atividade inibitória desta droga sobre superfamília de bombas de efluxo ABC (KOLACZKOWSKI; MICHALAK; MOTOHASHI, 2003).

O mecanismo da ação das fenotiazinas contra bombas de efluxo ainda não está totalmente compreendido, podendo ter ação de interação com as glicoproteínas-P (P-gp) das bombas (KOLACZKOWSKI; MICHALAK; MOTOHASHI, 2003) ou ação na membrana plasmática. Assim, os efeitos das fenotiazinas sobre membranas sugerem que a sua ação de inversão de MDR poderia, eventualmente, ser exercida, não por inibição

direta da P-gp, mas indiretamente por perturbar a matriz de fosfolípideo em que a P-gp está incorporada (NERDAL et al., 2000).

Brilhante et al. (2011), Brilhante et al. (2012) e Castelo-Branco et al. (2013) sugeriram que o mecanismo envolvido na resistência aos azólicos encontrado entre *Candida* spp. isolados a partir de animais de vida livre, que nunca tinham sido tratados com a droga antifúngica, é a superexpressão de bombas de efluxo, em resposta à exposição a estes animais e a sua microbiota levedura a poluentes ambientais. Embora a avaliação da resistência a antifúngicos tenha sido bastante estudada, ainda são poucos os estudos com isolados de ambientes aquático (BRANDÃO et al., 2010; BRILHANTE et al., 2011; MEDEIROS et al., 2008).

3 JUSTIFICATIVA

Existem muitos trabalhos com o monitoramento do perfil de sensibilidade a drogas de leveduras isoladas de amostras clínicas humanas. São escassos, porém, os trabalhos com leveduras isoladas do ambiente. Em pesquisa realizada por Medeiros et al. (2008), Brandão et al. (2010) e Brilhante et al. (2011), com amostras oriundas de ambientes aquáticos, foram obtidos elevado número de isolados de *Candida* spp. apresentando resistência *in vitro* a derivados azólicos. Medeiros et al. (2008) e Brandão et al. (2010) associaram o fenômeno de resistência à presença de efluentes de esgotos domésticos no corpo d'água, e alertaram ao risco dessas leveduras à saúde pública, enquanto que Brilhante et al. (2011) sugeriram que a presença de poluentes poderia estar relacionado com esse fenômeno.

Acredita-se que o fenômeno de resistência observado nessas cepas de *Candida* esteja relacionado às atividades antrópicas desenvolvidas na área estudada, uma vez que dejetos industriais e poluentes são capazes de alterar a expressão ou a composição gênica (MÜLLER et al., 2007; WĘGRZYN; CZYZ, 2003) e que a resistência a antifúngicos está principalmente associada às modificações genéticas dessa natureza (FENG et al., 2010). Ademais, fertilizantes, pesticidas e antifúngicos utilizados na agricultura também são implicados neste fenômeno (MÜLLER et al., 2007).

Vale destacar que o uso de animais sentinelas no isolamento de leveduras em ambientes aquáticos parece ser promissor. Ademais, no Brasil, a espécie de camarão *M. amazonicum* parece ter um papel central no monitoramento da microbiota de ambientes aquáticos (ANDRADE et al., 2010; BRILHANTE et al., 2011).

Ante o exposto, torna-se de fundamental importância avaliar o uso de leveduras do gênero *Candida* isoladas de camarões *M. amazonicum* e ecossistemas aquáticos como bioindicadores de poluição ambiental, por meio da análise quali-quantitativa da composição da microbiota e do monitoramento da resistência antifúngica.

4 HIPÓTESES

4.1. Existe resistência a derivados azólicos em leveduras isoladas de *Macrobrachium amazonicum* e da lagoa do Catú, Aquiraz, Ceará, Brasil.

4.2. A resistência antifúngica em leveduras do gênero *Candida* isoladas deste camarão e de seu ambiente aquático é mediada por bombas de efluxo.

4.3. O camarão *M. amazonicum* é um sentinela para o isolamento de cepas de *Candida* spp. resistentes aos derivados azólicos fluconazol e itraconazol em ecossistemas de água doce.

5 OBJETIVOS

5.1 Objetivo Geral

Analisar o perfil de sensibilidade antifúngico *in vitro* em leveduras isoladas de *Macrobrachium amazonicum* e ecossistemas aquáticos, assim como investigar o papel deste camarão no monitoramento ambiental.

5.2 Objetivos Específicos

- 1 Identificar e quantificar as leveduras presentes no trato digestório de *Macrobrachium amazonicum*, coletados na água da lagoa do Catú, Aquiraz, Ceará-Brasil.
- 2 Identificar e quantificar as leveduras presentes na água da lagoa do Catu, Aquiraz, Ceará-Brasil.
- 3 Estabelecer o perfil de sensibilidade à antifúngicos *in vitro*, das cepas isoladas de camarão de vida livre e da lagoa do Catú, ante a anfotericina B e derivados azólicos itraconazol e fluconazol.
- 4 Avaliar a atividade da bomba de efluxo nas cepas de *Candida* spp. resistentes aos derivados azólicos por meio da utilização dos moduladores da atividade de bombas Prometazina (Fenotiazina) e Haloperidol (Butirofenona).
- 5 Avaliar o papel do camarão de água doce *M. amazonicum* como carreadores de *Candida* spp. a partir do ambiente aquático por meio das técnicas moleculares M13-fingerprinting e RAPD-PCR, a partir da construção de dendrogramas na análise das bandas de DNA das cepas avaliadas.

6 CAPITULO 1

Surveillance of azole resistance among *Candida* spp. as a strategy for the indirect monitoring of freshwater environments

Vigilância da resistência aos azólicos em *Candida* spp. como uma estratégia para o monitoramento indireto de ambientes de água doce.

Water, Air, & Soil Pollution – Mini Review

Surveillance of azole resistance among *Candida* spp. as a strategy for the indirect monitoring of freshwater environments

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Running title: *Candida* spp. for monitoring aquatic environments

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Abstract

The growing pollution mainly caused by the discharge of industrial, sanitary and agricultural wastes has become one of the main current environmental issues. Thus, the use of bioindicators has become an important tool for investigating environmental imbalance. In this context, microorganisms have shown to be important for the identification of altered environments because of their ubiquity and their ability to grow in inhospitable habitats. Yeasts of the genus *Candida* are potential bioindicators because of their ability to survive in contaminated freshwater environments. Besides, they are more frequently recovered than fecal coliforms. It is noteworthy that the nonspecific activity of efflux pumps, which help in cellular detoxification processes, may be associated with the presence of chemical compounds in contaminated environments. Thus, the activity of efflux pumps may be the main mechanism involved in the resistance to azole derivatives in *Candida* spp. and the assessment of their activity may also be a tool for environmental monitoring. As a result, the phenotypical and molecular evaluation of this antifungal resistance in *Candida* species has been pointed as a promising tool for monitoring the quality of aquatic environments. Hence, the objective of this study was to collect and systematize data pointing to an alternative use of *Candida* spp. as bioindicators by assessing the occurrence of azole resistance among environmental *Candida* as a strategy to monitor the quality of freshwater environments.

Keywords: Yeasts, *Candida* spp., aquatic environments, azole resistance, environmental monitoring.

1 Introduction

Strategies to evaluate, control and develop tools that allow determining qualitatively the chemical, physical and biological risks to which ecosystems and human and animal health are exposed have become a demand from the international society, especially due to the appearance of substances with toxic and mutagenic properties (Hacon 2003).

The increasing of the chemical and metal contamination of fresh water ecosystems and its consequent impact on living organisms represent one of the most important current environmental issues (Medeiros et al. 2008; Mariano et al. 2009; Andrade et al. 2010; Buchberger 2011; Chiba et al. 2011). The evaluation of the environmental risk associated with a given anthropic activity allows to predict the occurrence of environmental damages and/or its consequence for human and environmental health. The main goal of analyzing environmental risks is to promote the self-sustainable development of populations, decreasing the deleterious impacts on ecosystems (Hacon 2003).

Two different approaches can be used to monitor the quality of aquatic environments: direct detection of chemical pollutants and metals, through various methods of analysis, including those of high-performance, using the mass spectrometer, and immunochemistry, using antibodies (APHA/AWWA/WEF 1998; Buchberger 2011; Van Dyk and Pletschke 2011); or indirect detection of pollutants through the use of sentinels and bioindicators, which are recovered from degraded environments or have a direct relationship between the degree of pollution and the number of isolates. The techniques for the direct detection of pollutants share some positive features such as high sensitivity and specificity, and fast results. However, high performance techniques are expensive and need skilled and specific techniques for each of the assayed compounds.

Furthermore, although enzymatic and immunochemical methods are simpler and cheaper techniques, they often lead to false results, and can be inhibited by a large number of compounds including heavy metals and organic compounds. In addition the commercially available kits are not specific for the use to analyze aquatic environments (Van Dyk and Pletschke 2011).

The use of sentinels and bioindicators have been encouraged since studies have demonstrated their applicability for monitoring different environments (Medeiros et al. 2008; Gerba 2009; Brandão et al. 2010; Mahmoud and Taleb 2013). In this context, bioindicator organisms should be useful for different types of environments, their density should have a direct correlation with the degree of environmental pollution, they should be a component of the microflora of long-living warm-blooded animals and should be analyzed or tested through simple methods (Gerba 2009). These requirements may pose an obstacle to the selection of good bioindicators organisms, which reinforces the need to search for alternatives for the indirect detection of pollutants.

Although yeasts of genus *Candida* are ubiquitous and do not completely fit the concept of bioindicator described above, these microorganisms are sensitive to environmental changes, presenting phenotypical and genotypical alterations, such as the overexpression of efflux pumps for cellular detoxification. Therefore, the use these microorganisms as bioindicators becomes a promising tool to monitor different types of aquatic habitats, for the presence of chemical and metal pollution, without the need of identifying the recovered strains to the species level, which would make this a faster, simpler and less expensive analysis (Brilhante et al. 2012; Castelo-Branco et al. 2013).

Thus, in this work, a collection and a systematization of data pointing to an alternative use of *Candida* spp. as bioindicators of the quality of freshwater environments, through the surveillance of azole resistance among environmental strain, were performed.

2 Sentinels for the isolation of bioindicator yeasts

The intestinal microbiota of aquatic invertebrates is similar to that of the environment where they are inserted and yeasts can be more easily recovered from these animals than directly from water (Hagler et al. 1995). Thus, these animals, especially bivalve mollusks and prawns, can be used to enhance the isolation of yeasts from aquatic environments, which in turn act as bioindicators for the analysis of the environmental health (Hagler et al. 1995; Kutty and Philip, 2008).

The term sentinel was used for the first time, during the 1950's, to describe bivalves that were used for detecting and mapping radioactivity (Washington 1984) and, since then, the program Mussel Watch has been used in several subsequent studies, involving the use of bioaccumulators (Beeby 2001). In general, sentinels are biological monitors that are able to accumulate pollutants without suffering significant side effects and are, mainly, used to measure the quantity of a given pollutant, to increase the sensitivity of a given analytical procedure and/or to simplify the interpretation of a complex sign of pollution. In this context, sentinel species can be classified into three major groups: 1) monitor species, whose biological functions are decreased by certain pollutants; 2) indicator species, which indicate the presence of an imbalance through the increase or decrease of their occurrence in the environment, and 3) accumulator species, which accumulate pollutants in their tissues without suffering significant damage, allowing the quantification of the bioavailable fraction of a given chemical substance in an ecosystem (Beeby 2001).

Different organisms have been suggested as sentinels, such as annelids (Barus et al. 2007), fish (Zorita et al. 2008), mollusks (Beeby 2001; Mazzia et al. 2011), bees

(Lambert et al. 2012), benthic macroinvertebrates (Brilhante et al. 2011; Chiba et al. 2011), amphibians (Hopkins 2007), birds (Brilhante et al. 2012) and mammals (Mariano et al. 2009). Among these species, invertebrates are particularly interesting because of their filtering characteristics and easy recovery from aquatic and terrestrial environments (Beeby 2001; Mazzia et al. 2011). The studies with these animals have addressed the tissue accumulation of heavy metals, hydrocarbons, pesticides and other pollutants (Mahmoud and Taleb 2013). Considering that these chemical compounds may cause alterations in the expression or in the sequence of specific genes (Keenan et al. 2007; Müller et al. 2007), which, in turn, (Kanafani and Perfect 2008) may lead to azole resistance, it seems plausible to think that the tissue accumulation of chemical compounds might increase the azole resistance rate among yeasts from the microbiota of sentinel animals.

Brilhante et al. (2012), for example, evaluated the susceptibility of *Candida* spp. isolated from the gastrointestinal tract of birds of prey and observed a high proportion of azole resistant strains, demonstrating the potential role of these birds as sentinels for the recovery of *Candida* spp. for environmental monitoring. The origin of this resistance, however, seems to be related to the exposure of these microorganisms to chemical compounds and heavy metals that are ingested by the animals with food and water. Concerning aquatic environments, Andrade et al. (2010) isolated from wild-harvested *M. amazonicum* different species of resistant bacteria and suggested that this resistance was associated with the promiscuous use of antibacterial drugs in shrimp and fish farming, representing a risk for human health. In this study, the relationship between the occurrence of antimicrobial resistance and the presence of pollutants in the aquatic environment is clear and strengthens the proposal of using *Candida* species as bioindicators, since the resistance mechanisms involving efflux pump are very similar

among bacteria and fungi (Alexander et al. 1999). Similarly, Brilhante et al. (2011) suggested that *M. amazonicum* is a good sentinel for monitoring antifungal resistance among environmental *Candida* spp., while these resistant *Candida* may be an important indicator for the presence of chemical compounds in a given aquatic environment. Since the environment where these animals were collected holds agricultural activities, which include the use of chemicals, as well as industrial effluents, domestic sewage and recreational activities, such as the use of boats and jetskis, contributing for the release of petroleum products in water bodies.

3 Yeasts and the relationship with altered aquatic environments

Different studies have shown a global tendency in using yeasts from the freshwater environment and from animals as a tool for assessing the environmental quality (Simard 1971; Sage et al. 1997; Hagler, 2006; Chen et al. 2009; Coelho et al. 2010). Although pathogenic yeast species, such as *C. albicans*, *C. parapsilosis* and *C. glabrata*, are target species for research involving polluted freshwater environments, they are not isolated in an expected frequency (Hagler 2006). The most commonly recovered yeasts from these environments belong to the genera *Candida*, *Cryptococcus*, *Rhodotorula*, *Saccharomyces* and *Trichosporon* (Hagler et al. 1995), with emphasis on the species *C. famata*, *R. mucilaginosa*, *T. beigelii* and *Cryptococcus laurentii*, which have been considered bioindicators of pollution in aquatic environments (Hagler 2006). These species deserve special mention, as they have been isolated in several studies, often surpassing the amount of the recovered fecal coliform isolates (Chun 1984; Arvanitidou et al. 2002; Medeiros et al. 2008; Hagler 2006; Coelho et al. 2010; Brilhante et al. 2011). However, some studies show that even under low anthropogenic influences, some

freshwater environments seem to be ideal for the development of these yeasts (Sláviková et al. 1992; Libkind et al. 2003). This fact falls back into the concept of bio-indicator, emphasizing the importance of developing phenotypical studies with these ubiquitous microorganisms, such as antifungal susceptibility assays, to validate their use as indicators of freshwater pollution.

Several authors have stated that the analysis of the yeast microbiota is a valid tool for the evaluation of the state of eutrophication in a given aquatic environment (Arvanitidou et al. 2002; Hagler 2006; Medeiros et al. 2008; Brandão et al. 2010; Brilhante et al. 2011). In the 1970's, the use of yeasts as bioindicators was proposed, based on a study developed in St. Lawrence river, in Canada. This study demonstrated that in areas located close to sewer, different yeast species grew abundantly and the number of recovered isolates and the frequency of recovery could be used as a parameter to evaluate pollution (Simard 1971). In this context, Rosa et al. (1990) investigated the occurrence of yeasts and fecal coliforms in five water collection stations in Olhos D'Água Lake, in the state of Minas Gerais, Brazil, and observed that in three stations, the number of recovered yeasts was higher than that of fecal coliforms, which suggested that yeasts could be better bioindicators of pollution in water sources.

In researches performed in temperate regions, such as Australia, carotenogenic yeasts, such as *Rhodotorula* spp., were important for the evaluation of the anthropic influence in the aquatic environment, considering that these yeasts presented high recovery rate from groundwater degraded by anthropogenic activity and associated environmental changes (Lategan et al. 2012). It is worth noting that these yeasts were also found in oligotrophic environments, under low anthropogenic influences (Libkind et al. 2003). Hence, the role of these yeasts as bioindicators can be discarded, based on the

concept of bioindicator. However, studies assessing the recovery rate and the occurrence of antifungal resistance among these microorganisms should be encouraged.

Mainly, yeasts may be used as bioindicators to evaluate environmental changes through two mechanisms. The first mechanism correlates the recovery rate of different yeast species to different environmental conditions, taking into account the presence of pollution sources, such as sewage and industrial effluents, while the second one evaluates the presence of phenotypical alterations in these microorganisms recovered from altered environments. These mechanisms substantiate the use of yeasts as bioindicators of changes in aquatic environments (Simard 1971; Rosa et al. 1990; Hagler et al. 1995; Hagler 2006; Medeiros et al 2008; Brandão et al. 2010; Brilhante et al. 2011).

4 *Candida* spp. as bioindicators of the quality of aquatic ecosystem

Researches involving the recovery of yeasts have demonstrated the omnipresence of the genus *Candida* and several species of this genus can be easily recovered from aquatic environments (Table 1). This genus has shown to be predominant, when considering the elevated number of isolates, as well as the high recovery rate, independently of the season (Raspor and Zurpan 2006). This fact becomes even more evident when the samples are from aquatic sources. Therefore, different researches propose the use of *Candida* spp. as environmental bioindicators (Hagler et al., 1995; Medeiros et al., 2008; Coelho et al., 2010; Brandão et al. 2010). In researches that aim at correlating environmental degradation to anthropic activities, such as domestic sewage and industrial and agricultural effluents, *Candida* spp. has been one of the species of interest, since it is commonly associated with the presence of human and animal feces (Hagler 2006; Brandão et al. 2010).

C. famata is the most commonly isolated species from aquatic sources and its isolation at high rates characterizes environmental degradation with domestic sewage (Medeiros et al. 2008; Brilhante et al. 2011). Additionally, *C. famata* has been strongly associated with the recovery of fecal coliforms, which demonstrates its role as an indicator of pollution in aquatic environments (Rosa et al. 1990; Medeiros et al. 2008). The predominance of *C. famata* in these environments may be related to its great capacity to resist low temperatures and salinity and osmolarity variations (Raspor and Zurpan 2006). In addition, besides indicating eutrophication, this species appears to be closely associated with the removal of pollutants, such as hydrocarbons, from contaminated water (Farag and Soliman 2011).

C. guilliermondii has also been recovered from aquatic environments, especially those eutrophized or contaminated with domestic sewage and industrial wastewater, as demonstrated by Medeiros et al. (2008), Brandão et al. (2010) and Brilhante et al. (2011). It has been suggested that *C. guilliermondii* is capable of using several carbon sources, including hydrocarbons, contributing for the bioremediation of petroleum contaminated soils and water (Papon et al. 2013). Therefore, the use of this species must be further studied because these characteristics may guide the definition of an exclusive phenotypical approach for monitoring polluted environments.

Medeiros et al. (2008), for example, evaluated the yeast diversity in lakes and rivers in Southeastern Brazil and found that the genus *Candida* was the one with the highest number of species and the greatest number of isolates, out of which 50% were resistant to itraconazole. Thus, based on these results, the authors suggested that in the investigated aquatic environments, the presence of azole resistant *Candida* spp. was associated with the anthropic activities to which these environments were submitted. Brandão et al. (2010), also evaluated the diversity of yeasts in three lakes in Southeastern

Brazil and observed a high resistance rate to amphotericin B, itraconazole and fluconazole among *Candida* species. Both authors emphasized the occurrence of antifungal resistance among environmental *Candida* strains and treated it as a potential threat to human health. However, this antifungal resistance should also be associated with the quality of freshwater environments, since it may be directly related to the overexpression of efflux pumps, as a consequence of the exposure to chemical and metal pollutants.

5 Antifungal resistance to azole derivatives as a strategy for environmental monitoring

As previously mentioned, it seems plausible to think that the development of azole resistance among *Candida* spp. is related to the anthropic activities performed in the studied areas, since antifungal resistance is mainly mediated by changes in gene expression and/or gene nucleotide sequence (Feng et al. 2010) and industrial wastes and pollutants are capable of promoting such alterations (Keenan et al. 2007; Müller et al. 2007).

In general, there are two main mechanisms involved in the development of resistance to azole derivatives. The first mechanism is associated with the development of active efflux pumps that are encoded by the genes *CDR1* and *CDR2*, which belong to the superfamily of the ATP binding cassette, and the gene *MDR1*, which belongs to the class of the major facilitators. The superexpression of these genes avoids the increase of drug concentration within the cell, hence, compromising its efficacy. Up-regulation of *CDR1* and *CDR2* leads to resistance to almost all azole antifungals, while up-regulation of *MDR1* leads to fluconazole resistance (Kanafani and Perfect 2008). The second resistance mechanism is associated with alterations in the enzyme lanosterol C14 α -

demethylase, target molecule of the azole derivatives, which is encoded by the gene *ERG11*. Superexpression or mutations in that gene decreases the susceptibility or leads to azole resistance (Kanafani and Perfect 2008; Manastir et al. 2009).

Out of these two resistance mechanisms, the unspecific action of transmembrane efflux pumps seems to be more common, as demonstrated in previous studies (Cannon et al. 2009; Brilhante et al. 2012; Castelo-Branco et al. 2013). It has been demonstrated that microorganisms resist the toxic effects of different types of solvents, petroleum derivatives and mutagenic agents through the action of these efflux pumps, which export to the extracellular environment several substrates, such as heavy metals and chemical derivatives, including cadmium, arsenate, hexane, toluene, xylene, and others (Kieboom et al. 1998; Bruins et al. 2000). Additionally, efflux pumps are also responsible for several biological phenomena, acting in vital cellular processes, such as the secretion of peptides involved in cellular communication, pheromones, mitochondrial regulation, stress adaption and detoxification (Fig. 1) (Jungwirth and Kuchler 2006).

In this context, Brilhante et al. (2011), Brilhante et al. (2012) and Castelo-Branco et al. (2013) suggested that the mechanism involved in the azole resistance found among *Candida* spp. recovered from free-ranging animals that had never been treated with antifungal drugs was the overexpression of efflux pumps, as a response to the exposure of these animals and their yeast microbiota to environmental pollutants.

Thus, the evaluation of the yeast microbiota of a given aquatic environment and the assessment of the antifungal susceptibility of these yeasts seem particularly interesting, since the exposure of these microorganisms to chemical compounds interferes with yeast cell physiology, which may lead to azole resistance, as a consequence of alterations in gene expression and gene composition (Feng et al. 2010). In addition, the phenotypical evaluation of the antifungal susceptibility of *Candida* spp. from

environmental or animal sources seems particularly promising as a tool for environmental monitoring because *Candida* spp. are important biodegraders breaking down or absorbing chemical compounds, such as hydrocarbons and heavy metals, contributing to the bioremediation and detoxification of contaminated water (Farag and Soliman 2011), which decreases the concentration of pollutants in water sources hence interfering with the sensitivity of the direct detection of chemical compounds. However, in order to obtain reliable results and validate this phenotypical analysis, some aspects need to be better understood, such as the types and the concentration of the pollutants and the time of exposure necessary to promote antifungal resistance in these yeasts.

6 Final Considerations

Maintaining the environmental health has been one of the greatest concerns of the 21st century. Considering that yeasts, particularly *Candida* spp., have been associated with polluted freshwater environments, it is essential to standardize the use of these organisms for monitoring freshwater environments. Even though these yeasts are ubiquitous, monitoring the occurrence of efflux-mediated azole resistance could be considered an applicable strategy for the use of *Candida* spp. as a bioindicator of freshwater pollution. The presence of chemical pollutants in aquatic ecosystems where these yeasts are inserted increase efflux activity to decrease the intracellular concentration of chemicals compounds, avoiding damages in yeast cells and promoting the survival of *Candida* spp. It is important to emphasize that the increased activity of efflux pumps leads to azole resistance and the surveillance of this resistance among *Candida* spp. may act as a phenotypical strategy for monitoring aquatic environments.

In this context, we strongly believe that assessing the occurrence of azole resistance among *Candida* spp. from freshwater environments is a promising tool for this purpose. Thus, further studies must be conducted to better understand the relationship between the development of azole resistance and the presence of chemical pollutants in water sources.

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Table 1 – Main *Candida* species isolated from aquatic environments.

Species	Environment	Frequency(%)	References
<i>Candida famata</i>	Estuary	40-90	Chun 1984
	Estuary	6	Soares et al. 1997
	Sea water	66	Valdes-Collazo et al. 1987
	Estuary	12	Coelho et al. 2010
	Fresh water*	-	Medeiros et al. 2008
	Fresh water	-	Brandão et al. 2010
	Estuary	-	Almeida 2005
	Sea water	-	Butinar et al. 2005
	Fresh water	23	Brilhante et al. 2011
<i>C. sake</i>	Estuary	12	Chun 1984
	Sea water	13	Loureiro et al. 2005
<i>C. tropicalis</i>	Estuary	12	Chun 1984
	Estuary	8	Soares et al. 1997
	Fresh water	8	Brilhante et al. 2011
<i>C. parapsilosis</i>	Estuary	12	Chun 1984
	Estuary	5	Soares et al. 1997
	Estuary	8	Valdes-Collazo et al. 1987
	Estuary	9	Coelho et al. 2010
	Fresh water	-	Medeiros et al. 2008
	Fresh water	-	Brandão et al. 2010
	Sea water	-	Butinar et al. 2005
	Sea water	-	Gadanhó and Sampaio, 2005
	Sea water	13	Loureiro et al. 2005
<i>C. glabrata</i>	Estuary	5	Soares et al. 1997
<i>C. krusei</i>	Estuary	8	Soares et al. 1997
<i>C. albicans</i>	Estuary	46	Valdes-Collazo et al. 1987
	Fresh water	15	Valdes-Collazo et al. 1987
	Fresh water	-	Sage et al. 1997
	Sea water	5	Loureiro et al. 2005
	Fresh water	31	Valdes-Collazo et al. 1987
<i>C. guilliermondii</i>	Estuary	8	Coelho et al. 2010
	Fresh water	-	Medeiros et al. 2008
	Fresh water	-	Brandão et al. 2010
	Sea water	-	Butinar et al. 2005
	Fresh water	11,5	Brilhante et al. 2011
	Sea water	-	Gadanhó and Sampaio, 2005
	Estuary	9	Coelho et al. 2010
<i>C. zeylanoides</i>	Estuary	9	Coelho et al. 2010
<i>C. melibiosica</i>	Fresh water	-	Medeiros et al. 2008
<i>C. sorbophila</i>	Fresh water	-	Medeiros et al. 2008
<i>C. catenulata</i>	Sea water	17	Loureiro et al. 2005
<i>C. fennica</i>	Sea water	17	Loureiro et al. 2005
<i>C. maltosa</i>	Fresh water	12,5	Sláviková and Vadkertiová, 1997

*Environment: fresh water = river and lakes

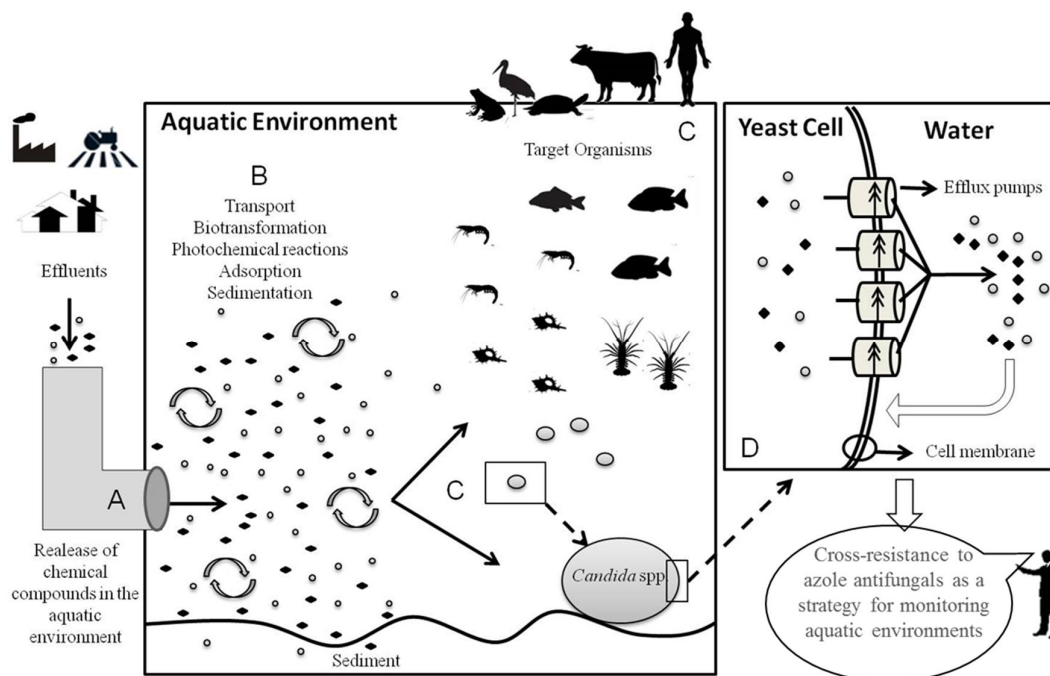


Figure 1: A: Release of chemical compounds in the aquatic environment from industrial, agricultural and residential waste; B: Chemical compounds are transported and go through transformation, adsorption and sedimentation in the aquatic environment; C: Several organisms (mammals, birds, reptiles, amphibians, fish, invertebrates and microorganisms) are exposed to these chemical compounds; D: These compounds penetrate *Candida spp.* cells, which, in turn, increase efflux activity to decrease the intracellular concentration of chemicals compounds, avoiding cellular damages and promoting the survival of *Candida* cells.

7 CAPÍTULO 2

Macrobrachium amazonicum: an alternative for microbiological monitoring of aquatic environments in Brazil

Macrobrachium amazonicum: uma alternativa para o monitoramento microbiológico de ambientes aquáticos no Brasil

Ciência Rural – Artigo Científico

Macrobrachium amazonicum: an alternative for microbiological monitoring of aquatic environments in Brazil

Macrobrachium amazonicum uma alternativa para o monitoramento microbiológico de ambientes aquáticos no Brasil

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ABSTRACT

This study aimed to evaluate the role of the Amazon River prawn, *Macrobrachium amazonicum*, as carrier of *Candida* spp., by analyzing the correlation between *Candida* spp. from these prawns and their environment (surface water and sediment), through M13-PCR fingerprinting and RAPD-PCR. For this purpose, 27 strains of *Candida* spp. were evaluated. These strains were recovered from the gastrointestinal tract of adult *M. amazonicum* (7/27) from Catú Lake, Ceará State, Brazil and from the aquatic environment (surface water and sediment) of this lake (20/27). Molecular comparison between the strains from prawns and the aquatic environment was conducted by M13-PCR fingerprinting and RAPD-PCR, utilizing the primers M13 and OPQ16, respectively. The molecular analysis revealed similarities between the band patterns of eight *Candida* isolates with the primer M13 and 11 isolates with the primer OPQ16, indicating that the same strains are present in the digestive tract of *M. amazonicum* and in the aquatic environment where these prawns inhabit. Therefore, these prawns can be used as sentinels for environmental monitoring through the recovery of *Candida* spp. from the aquatic environment in their gastrointestinal tract.

Key words: *Macrobrachium amazonicum* prawn, environmental sentinel, *Candida* spp., pollution, monitoring.

RESUMO

Este estudo teve como objetivo avaliar o papel do camarão *Macrobrachium amazonicum* como carreador de *Candida* spp. do ambiente aquático, por meio da análise da correlação entre *Candida* spp. isoladas desse camarão e do seu ambiente (água de superfície e sedimento) através das técnicas de M13-PCR fingerprinting e RAPD-PCR. Para tanto, 27 cepas de *Candida* spp. foram avaliadas. Essas cepas foram recuperadas a partir do trato gastrointestinal de *M. amazonicum* adultos (7/27), oriundos da lagoa do Catú, Ceará, Brasil, e do meio aquático (águas superficiais e sedimentos) desse lago (20/27). A comparação molecular entre as cepas de camarões e o ambiente aquático foi conduzida por M13-PCR fingerprinting e RAPD-PCR, utilizando os iniciadores M13 e OPQ16, respectivamente. A análise molecular revelou semelhanças entre os padrões de bandas de oito isolados de *Candida* com o iniciador M13 e 11 isolados com o primer OPQ16, indicando que elas estão presentes no trato digestivo de *M. amazonicum* e no ambiente aquático, onde esses camarões habitam. Portanto, essa espécie de camarão pode ser usada como sentinela para monitoramento ambiental através da recuperação de *Candida* spp. do ambiente aquático, a partir do seu trato gastrointestinal.

Palavras-chave: camarão *Macrobrachium amazonicum*, sentinela ambiental, *Candida* spp, poluição, monitoramento.

INTRODUCTION

Several studies have identified *Candida* spp. as potential biological indicators of environmental degradation (MEDEIROS et al., 2008; BRILHANTE et al., 2011, 2012; CASTELO-BRANCO et al., 2013), particularly in samples obtained from aquatic sources (BUTINAR et al., 2005; MEDEIROS et al., 2008). In these studies, the isolation of this genus was greater than that of other microorganisms, including bacteria, demonstrating the potential use of this yeast for environmental monitoring.

Monitoring aquatic environments requires an adequate water sampling technique, including the selection of representative collection sites and considering environmental factors, such as seasonality, temperature, the water column and the presence of affluent or effluent waters (APHA/AWWA/WEF, 1998). These requirements may represent an obstacle for the adequate monitoring of fresh water environments, because of the large number of samples needed. Hence, it is important to seek alternatives to facilitate monitoring of aquatic ecosystems. In this context, the use of aquatic crustaceans has been reported as a reliable alternative for that purpose, especially because of their feeding habits (filter feeding) and benthic behavior, as described by VIRGA et al. (2007) and BRILHANTE et al. (2011).

More recently, BRILHANTE et al. (2011) performed a research with the freshwater prawn *M. amazonicum* (Amazon River prawn) in captivity and from the natural environment for the isolation of yeasts and *Candida* was the most isolated genus, showing that it belongs to the gastrointestinal microbiota of these animals. In addition, these authors suggested that these prawns may be an important environmental sentinels if they harbor in their gastrointestinal tract *Candida* spp. from the aquatic environment. Thus, in light of these findings and considering the wide distribution of the species *M.*

amazonicum in South America, the objective of the present study was to evaluate the role of these prawns as carriers of *Candida* spp. from the aquatic environment.

MATERIALS AND METHODS

Microorganisms

In this study, 27 strains of *Candida* spp. were analyzed, out of which seven were recovered from wild-harvested *M. amazonicum*, while 20 were recovered from the aquatic environment and were deposited in our culture collection. It is important to emphasize that the analyzed *Candida* strains, from animal and environmental sources, were simultaneously recovered.

Of the 20 environmental strains, 13 were isolated from surface water (SW) and seven from sediment (S). These strains belong to the culture collection of the Specialized Medical Mycology Center of the Federal University of Ceará, where they are kept on 2% Sabouraud dextrose agar. They were manipulated under level II biosafety procedures.

Candida spp. from the aquatic environment were obtained from Catú Lake, which is located at the municipality of Aquiraz, Ceará state, Brazil, about 35 kilometers from the state capital. It is delimited by the UTM coordinates 0567000E, 9561273N and 0575000E, 9569000N. It is a rich freshwater body with mangrove areas that have been degraded by uncontrolled occupation of the surrounding area and pollution with residues from industrial, commercial and farming activities (BRILHANTE et al., 2011).

Water samples were collected, according to MEDEIROS et al. (2008). Then, four collection sites were included, as follows: recreational area and prawn collection site (point 1, 3°55'59.79" S and 38°21'50.10" W), agricultural wastewater (point 2, 3°55'47.25" S and 38°22'14.16" W), industrial wastewater (point 3, 3°56'03.70" S and

38°22'25.15" W) and residential area (point 4, 3°56'56.72" S and 38°22'31.57" W). Two water samples (SW and S) were monthly collected from each collection site, during one year (from March 2011 to February 2012). Overall, a total of 96 water samples were obtained for this study.

Adults of *M. amazonicum* were monthly harvested from Catú Lake (point 1) in the same period as the water samples. Afterwards, the digestive tracts of 10 individuals were removed, placed in sterile slants containing sterile saline and treated as one single sample (BRILHANTE et al., 2011). Overall, 12 collections were performed. This study was previously approved by the Chico Mendes Institute for Conservation of Biodiversity/Biodiversity Authorization and Information System – SISBIO, under the number 28175-1.

Microbiological processing

Samples were processed in a biosafety level II cabinet and were seeded on 2% Sabouraud agar plus chloramphenicol (0.5g L^{-1}), in Petri dishes for primary isolation. Water samples were processed according to MEDEIROS et al. (2008) with some modifications. Briefly, a 100- μL aliquot of the SW samples was spread on the medium, after homogenization, while the S samples were processed, after centrifuging for 20 minutes at 3,000rpm. Then, the supernatant was removed and the substrate was suspended again in 2mL of a sterile 0.9% solution of NaCl. Afterwards, the suspension was agitated in a vortex mixer for 3 minutes and left to rest for 30 minutes at 25°C. Next, 100- μL aliquots of the supernatant of each sample were seeded on the culture medium. The digestive tracts of adult prawns were processed as described by BRILHANTE et al. (2011) and seeded onto the culture medium. The inoculated Petri dishes containing the culture media were incubated at 25°C, for 10 days, and were daily observed (BRILHANTE et al., 2011).

The yeast colonies were identified through specific morphological and biochemical tests, including growth on chromogenic medium (CHROMagar Candida, BD, USA), micromorphology on cornmeal-Tween 80 agar, carbohydrate and nitrogen assimilation and urease production (BRILHANTE et al., 2011), and the results were interpreted according to (DE HOOG et al., 2000). Strains that presented dubious identity were also identified through VITEK II automated system (BioMérieux, USA). Additionally, the susceptibility of these microorganisms to amphotericin B, fluconazole and itraconazole was evaluated through broth microdilution method. Minimum inhibitory concentrations (MIC) of >1 , ≥ 64 , $\geq 1 \mu\text{g mL}^{-1}$ were considered resistant to amphotericin B, fluconazole and itraconazole, respectively (CLSI, 2008).

- M13-PCR fingerprinting and OPQ-16 RAPD

The DNA from the strains was extracted after 48 hours of growth on potato dextrose agar, according to the methodology described by CASTELO-BRANCO et al. (2013).

For molecular comparison between the *Candida* isolates from the aquatic environment (SW and S) and from prawns, the PCR-fingerprinting technique was used, according to the method described by CASTELO-BRANCO et al. (2013), using the single primer M13 (59-GAGGGTGGCG GTTCT-39) and the PCR mix (25 μL), containing 10mM of Tris/HCl (pH 8.3), 50mM of KCl, 1.5mM of MgCl_2 , 0.2mM of dNTPs, 0.15mM of the primer, 2.5U of Taq polymerase (MBI Fermentas) and 25ng of yeast DNA. The RAPD reactions were performed with the primer OPQ16 (5' AAGAGCCCGT3'), according to the method described by CASTELO-BRANCO et al. (2013). The RAPD reaction was carried out with a total volume of 10 μL , containing 50ng of genomic DNA, 1X buffer, 1mM of MgCl_2 , 2pmol of primer, 0.5mM each of deoxynucleoside triphosphate and 1 U μL^{-1} of Hot Start Taq polymerase.

Dice similarity coefficient was measured and a dendrogram was obtained through the use of the Unweighted Pair Group Method with Arithmetic Average (UPGMA), through the software BioNumerics (version 6.6), resulting in the analysis of clusters and measure of relatedness among isolates.

RESULTS

Data referring to the identity, the origin and the antifungal susceptibility profile of the recovered *Candida* strains are listed in table 1. Five to eight DNA bands were generated through the M13-PCR fingerprinting, while three to ten DNA bands were generated through RAPD-PCR with the primer OPQ16. The molecular analysis employing both techniques revealed strong similarities between the DNA band patterns of the isolates belonging to the same *Candida* species. For the primer M13, eight isolates of *Candida* spp. with 100% band similarity were obtained, while with the primer OPQ16, 11 isolates were obtained with 100% band similarity (Figure 1).

The M13-PCR fingerprinting identified 100% similarity between two *C. tropicalis* strains from prawn (n=1) and sediment (point 3, n=1); four *C. famata* strains from prawns (n=2) and surface water (points 1 and 3; n=2) and two *C. ciferrii* strains from prawns (n=1) and sediment (point 1, n=1). In turn, the RAPD-PCR with the primer OPQ16 allowed identifying 100% similarity between two *C. guilliermondii* strains from surface water (points 1 and 4); five *C. famata* strains, two from prawn (n=1) and surface water (point 1, n=1) and three from surface water (points 1 and 2); two strains of *C. parapsilosis* from prawn (n=1) and surface water (point 4, n=1) and two *C. ciferrii* strains from prawn (n=1) and sediment (point 1, n=1) (Figure 1).

DISCUSSION

This study demonstrated the similarity among *Candida* spp. isolated from wild-harvested prawns and the aquatic environment where the animals inhabit, including surface water and sediment. The molecular analysis through M13-PCR fingerprinting and RAPD-PCR with OPQ-16 allowed evaluating this correlation, since these techniques generated varied band patterns among different *Candida* species and similar ones within the same species, thus presenting desirable and reliable results. In the present study, the primer OPQ16 was used to complement the results obtained through the M13-PCR fingerprinting and it generated a greater variety of DNA bands and identified a greater number of strains with 100% of similarity.

The recovered *Candida* species were simultaneously isolated from prawns and the aquatic environment and some of these isolates presented 100% of similarity, even when recovered from different collection points. Thus, it was demonstrated that *M. amazonicum* contains in its gastrointestinal tract a representative cross-section of *Candida* spp. that colonize the water and the substrate where they live.

In addition, three sets of azole resistant strains were observed among the isolates from prawns and aquatic environment that presented 100% of similarity. These findings are in accordance with those of BRILHANTE et al. (2011), who observed that 28.6% of the *Candida* spp. recovered from the intestinal tract of wild-harvested *M. amazonicum* isolated from Catú Lake were resistant to azole antifungals. Considering that the main mechanism of azole resistance among *Candida* spp. is the overexpression of efflux pumps (FENG et al., 2010), which is possibly related to the exposure of these microorganisms to chemical compounds, as an unspecific mechanism of cellular detoxification (JUNGWIRTH & KUCHLER, 2006), we strongly believe that *Candida* spp. could be

used as indicators of environmental pollution, through the phenotypical assessment of their *in vitro* susceptibility profile.

Crustaceans accumulate pollutants in their tissues, such as hydrocarbons, pesticides and heavy metals (YILMAZ & YILMAZ, 2007), which might increase the azole resistance rate among yeasts from the microbiota, due to the overexpression of efflux pumps (KEENAN et al., 2007; MÜLLER et al., 2007), as a consequence of the chronic exposure to these chemical compounds. In this context, the use of this prawn as a sentinel for the isolation of *Candida* spp. seems potentially advantageous.

CONCLUSION

In conclusion, based on the obtained results, the use of *M. amazonicum* as a sentinel for the isolation of *Candida* spp. from aquatic environments is an interesting alternative for evaluating the environmental quality, considering that these animals harbor yeasts from the environment in their gastrointestinal tract. Additionally, due to their capacity to accumulate chemical pollutants in their tissues, they simulate the environmental conditions to which these yeasts are exposed, potentially contributing for monitoring the presence of resistant *Candida* spp. in the environment.

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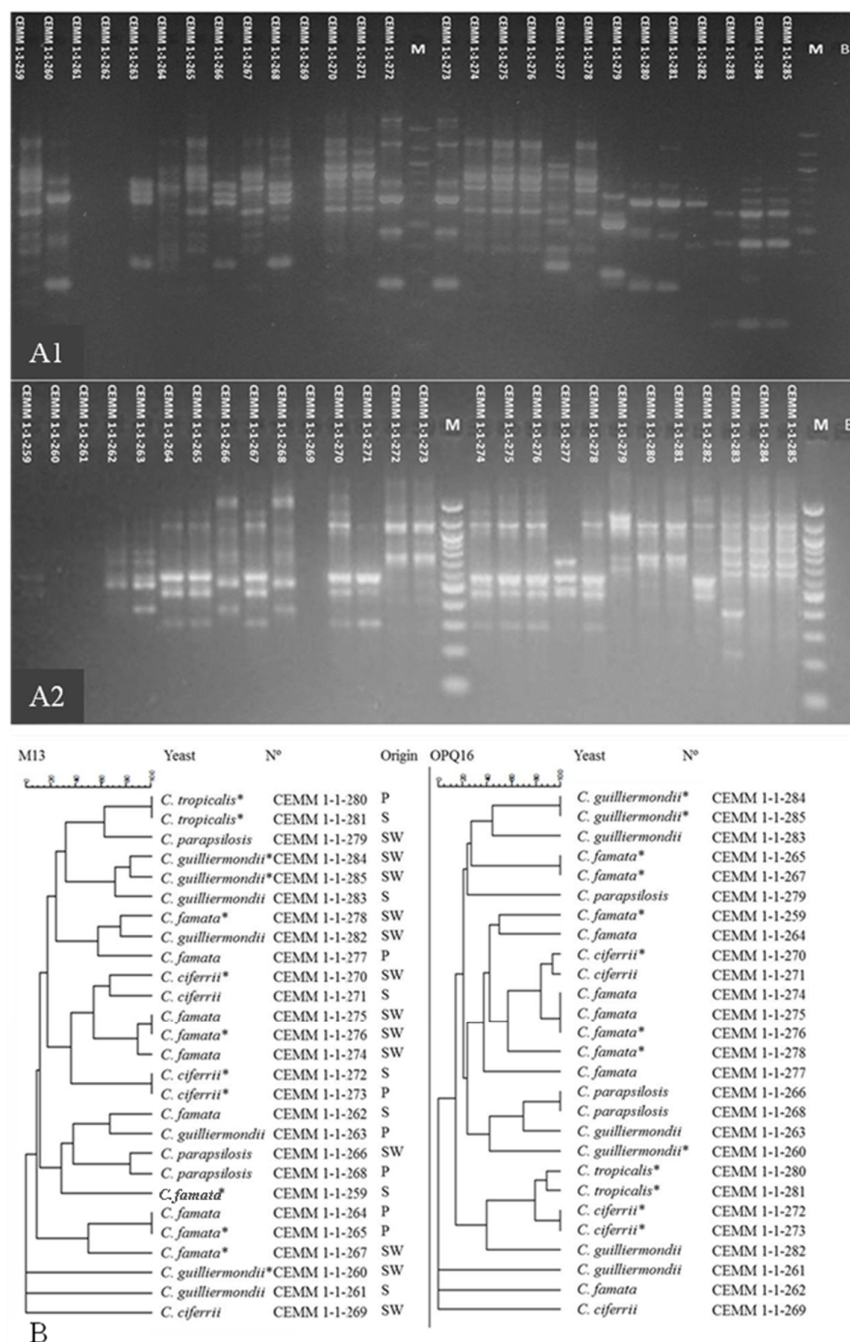


Figure 1 - Dendrograms resulting from the analysis of 27 isolates of *Candida* spp. obtained from the gastrointestinal tract of *Macrobrachium amazonicum* (n=7) and the natural environment (n=20) through M-13-fingerprinting and RAPD-PCR with primer OPQ16. P: prawn; SW: surface water; S: sediment. *indicates antifungal resistance. Dendrograms generated by the BioNumerics program (Applied Math, Inc.).

Table 1 - Species, access number, origin, isolation period and antifungal susceptibility of 27 *Candida* spp. isolates used for molecular analysis.

Species	Access number	Origin	Collection point	Period	Resistance*
<i>C. famata</i>	CEMM 1-1-259	Sediment	Point 3	October 2011	FLC/ITC
<i>C. guilliermondii</i>	CEMM 1-1-260	Surface Water	Point 4	November 2011	ITC
<i>C. guilliermondii</i>	CEMM 1-1-261	Sediment	Point 4	November 2011	S
<i>C. famata</i>	CEMM 1-1-262	Sediment	Point 3	November 2011	S
<i>C. guilliermondii</i>	CEMM 1-1-263	Prawn	Point 1	December 2011	S
<i>C. famata</i>	CEMM 1-1-264	Prawn	Point 1	November 2011	S
<i>C. famata</i>	CEMM 1-1-265	Prawn	Point 1	November 2011	ITC
<i>C. parapsilosis</i>	CEMM 1-1-266	Surface Water	Point 4	November 2011	S
<i>C. famata</i>	CEMM 1-1-267	Surface Water	Point 1	August 2011	ITC
<i>C. parapsilosis</i>	CEMM 1-1-268	Prawn	Point 1	March 2011	S
<i>C. ciferrii</i>	CEMM 1-1-269	Surface Water	Point 3	October 2011	S
<i>C. ciferrii</i>	CEMM 1-1-270	Surface Water	Point 1	August 2011	ITC
<i>C. ciferrii</i>	CEMM 1-1-271	Sediment	Point 4	November 2011	S
<i>C. ciferrii</i>	CEMM 1-1-272	Sediment	Point 1	October 2011	FLC/ITC
<i>C. ciferrii</i>	CEMM 1-1-273	Prawn	Point 1	October 2011	FLC/ITC
<i>C. famata</i>	CEMM 1-1-274	Surface Water	Point 3	November 2011	S
<i>C. famata</i>	CEMM 1-1-275	Surface Water	Point 3	May 2011	S
<i>C. famata</i>	CEMM 1-1-276	Surface Water	Point 1	August 2011	FLC/ITC
<i>C. famata</i>	CEMM 1-1-277	Prawn	Point 1	December 2011	S
<i>C. famata</i>	CEMM 1-1-278	Surface Water	Point 4	October 2011	FLC
<i>C. parapsilosis</i>	CEMM 1-1-279	Surface Water	Point 1	May 2011	S
<i>C. tropicalis</i>	CEMM 1-1-280	Prawn	Point 1	October 2011	FLC/ITC
<i>C. tropicalis</i>	CEMM 1-1-281	Sediment	Point 3	October 2011	FLC/ITC
<i>C. guilliermondii</i>	CEMM 1-1-282	Surface Water	Point 4	December 2011	S
<i>C. guilliermondii</i>	CEMM 1-1-283	Sediment	Point 3	December 2011	S
<i>C. guilliermondii</i>	CEMM 1-1-284	Surface Water	Point 4	August 2011	ITC
<i>C. guilliermondii</i>	CEMM 1-1-285	Surface Water	Point 1	December 2011	FLC/ITC

*All strains were susceptible to amphotericin B, S: susceptible to all tested antifungal drugs (amphotericin B, fluconazole and itraconazole); FLC: resistance to fluconazole; ITC: Resistance to itraconazole.

8 CAPÍTULO 3

Azole resistance in Candida spp. from water: an efflux-pump mediated mechanism

Resistência à azólicos em *Candida* spp. oriundas de água: um mecanismo mediado por bomba de efluxo

Brazilian Journal of Microbiology – Research Paper**Azole resistance in *Candida* spp. from water: an efflux-pump mediated mechanism**

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Running title: *Candida* spp. for monitoring aquatic ecosystems

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Abstract

Even though azole resistance has been reported among yeasts recovered from aquatic environments, no studies have reported the mechanisms underlying this antifungal resistance. Thus, the present study aimed at investigating the occurrence of antifungal resistance among yeasts recovered from an aquatic environment, and assessing the efflux pump activity of the azole resistant strains, to better understand the mechanisms of resistance to this group of drugs. For this purpose, water and sediment samples were collected from Catú Lake, Ceará, Brazil, from March 2011 to February 2012. The obtained yeasts were identified through morphological and biochemical analyses. Overall, 46 isolates were recovered, of which 37 were *Candida* spp., four *Trichosporon asahii*, three *Cryptococcus laurentii*, one *Rhodotorula mucilaginosa* and one *Kodamaea ohmeri*. These isolates were submitted to broth microdilution assay with amphotericin B, itraconazole and fluconazole, according to CLSI. The MICs for amphotericin B, itraconazole and fluconazole were 0.03125 to 2 µg/mL, 0.0625 to ≥16 µg/mL and 0.5 to ≥64 µg/mL, respectively, with 13 azole resistant strains. When efflux pump inhibitors were added, a reduction in the azole MICs was observed, leading to the phenotypical reversion of the azole resistance. This finding suggests that the azole resistance among environmental *Candida* spp. is most likely associated with the overexpression of these pumps.

Keywords: Aquatic environments, yeast microbiota, *Candida* spp., antifungal resistance

1. Introduction

The quali-quantitative analysis of the yeast microbiota has been indicated as a promising tool to assess the eutrophication status of aquatic systems (Arvanitidou et al., 2002; Coelho et al., 2010; Hagler et al., 1995; Libkind et al., 2003; Nagahama, 2006; Medeiros et al., 2008). Medeiros et al. (2003), for example, studied the diversity of yeasts in lakes and rivers in Southeastern Brazil and the genus *Candida* accounted for the largest number of isolates, out of which 50% were resistant to itraconazole and 11% were resistant to fluconazole. More recently, our group conducted a study using the freshwater prawn *Macrobrachium amazonicum* (Amazon River prawn) collected in the natural environment and observed that 33.3% of the *Candida* spp. isolates from this prawn were resistant to fluconazole and itraconazole (Brilhante et al., 2011).

However, none of the researches mentioned above investigated the mechanisms underlying the azole resistance observed among *Candida* strains from aquatic environments. It is well known that one of the main mechanisms of azole resistance among *Candida* spp. is the increased activity of efflux pumps, which are codified by the genes *CDR1* and *CDR2*, belonging to the superfamily of the ATP binding cassette, and by the gene *MDR1*, belonging to the major facilitator class. The superexpression of these genes and the subsequent increase in the activity of these pumps prevents the accumulation of the drug inside the cell, at the site of action, impairing its efficacy (Jungwirth and Kuchler, 2006). The upregulation of *CDR1* and *CDR2* confers resistance to nearly all azoles, while that of *MDR1* provides specific resistance to fluconazole (Kanafani and Perfect, 2008). Thus, the present study was undertaken to investigate the occurrence of antifungal resistance among yeasts recovered from an aquatic environment, and assessing the efflux pump activity in the azole resistant strains.

2. Methods

2.1 Study site and Collections of the biological material

Catú Lake, located in the municipality of Aquiraz, Ceará State, Brazil (UTM coordinates 0567000E, 9561273N and 0575000E, 9569000N), is a rich freshwater body with mangrove areas that shelter a large number of animal species. However, due to the uncontrolled occupation of the surrounding area, the waters of this lake are mainly used for human and animal consumption, for industrial, commercial and farming activities and for leisure activities, such as boat excursions (Gomes et al., 2008).

Water samples were obtained monthly, from March 2011 to February 2012, completing a total of 12 collections, according to the method described by Medeiros et al. (2008), with some modifications. The water samples were obtained with a 1-liter Van Dorn bottle, which was rinsed three times with water from each collection site, before performing the water collection from four sites: recreational area point (point 1, 3°55'59.79" S and 38°21'50.10" W), agricultural wastewater point (point 2, 3°55'47.25" S and 38°22'14.16" W), industrial wastewater point (point 3, 3°56'03.70" S and 38°22'25.15" W) and Catú River confluence point (point 4, 3°56'56.72" S and 38°22'31.57" W) (Fig. 1). Two samples were collected at each point, one from the surface (SW sample) and the other from the bottom, including sediment (S sample). The study was approved by the Chico Mendes Institute for Conservation of Biodiversity/Biodiversity Authorization and Information System – SISBIO, under the process number 28175-1.

2.2 Mycological processing

The samples were taken to the laboratory and processed in a biological safety level 2 laminar flow cabinet. For each sample, Sabouraud agar supplemented with chloramphenicol (0.5 g/L) was used as the culture medium for primary isolation, in Petri dishes. For the SW samples, after homogenization, a 100- μ L aliquot was spread on the medium. The S samples were processed, as follows: the samples were centrifuged for 20 minutes at 3,000 rpm, the supernatant was removed and the sediment was resuspended in 2 mL of sterile 0.9% NaCl solution. Then, the suspension was agitated in a vortex mixer for 3 minutes and left to rest for 30 minutes at 25 °C. Afterwards, 100- μ L aliquots of the supernatant of each sample were spread on the culture medium. The inoculated Petri dishes were incubated at 25 °C for 10 days, with daily observations to note the microbiological growth. The colony forming units (CFUs) were also counted in all the inoculated dishes.

2.3 Yeast Identification

Initially, the colonies that appeared to be yeasts were Gram stained and observed under a light microscope (40X) to check for the presence of blastoconidia, hyphae or pseudohyphae and to exclude bacterial contamination. Then, the yeast colonies were identified through specific macromorphological and micromorphological characteristics, including growth on chromogenic medium for the identification of mixed colonies, and biochemical tests, such as carbohydrate and nitrogen assimilation and urease production. VITEK 2™ microbial identification system (bioMérieux, USA) was used in case of dubious identification, to aid the identification procedure (Brilhante et al., 2011).

2.4 *In vitro* antifungal susceptibility testing

The antifungal minimum inhibitory concentrations (MICs) against these microorganisms were determined through broth microdilution method, as described by the Clinical and Laboratory Standards Institute (CLSI, 2008). Three drugs were tested against the isolates: amphotericin B (Sigma Chemical Corp.), itraconazole (Janssen Pharmaceutica, Belgium) and fluconazole (Pfizer, Brazil). Inocula of all tested isolates were prepared from 1-day old cultures grown on potato dextrose agar at 35 °C with RPMI 1640 medium, with L-glutamine (HiMedia Laboratories), buffered to pH 7 with 0.165M morpholinepropanesulfonic acid and were adjusted to a final concentration of $0.5\text{--}2.5 \times 10^3$ cells mL⁻¹ (Brilhante et al., 2011; CLSI, 2008). The microdilution plates were incubated at 35 °C, for 48 hours, and were visually read (CLSI, 2008). For each isolate, drug-free and yeast-free controls were included and all the isolates were tested in duplicate. As quality control, for each test performed, *C. parapsilosis* ATCC 22019 was included. The MIC of azole derivatives was defined as the lowest drug concentration capable of inhibiting 50% of growth, when compared with the growth control well. For amphotericin B the MIC was the lowest concentration at which no growth was observed. Isolates with MICs >1, ≥ 1 and ≥ 64 µg/mL were considered resistant to amphotericin B, itraconazole and fluconazole, respectively (CLSI, 2008).

2.5 Analysis of the efflux pump activity in the azole resistant *Candida* isolates

A phenotypical assay of modulation of efflux pumps was carried out, based on the method used by Castelo-Branco et al., (2013). Initially, the azole resistant *Candida* strains (13/37) were tested against two efflux pump inhibitors, promethazine (Kolaczowski et

al., 2003) and haloperidol (Iwaki et al., 2006) by microdilution method, obtaining average MIC values of 98 µg/mL and 80 µg/mL, respectively. Subsequently, susceptibility test was performed with itraconazole and fluconazole, according to the methodology described above, but, sub-inhibitory concentrations of promethazine (12 µg/mL) and haloperidol (10 µg/mL) were added to the final fungal inocula. Fluconazole was used in combination with promethazine and haloperidol, while itraconazole was tested only with promethazine.

2.6 Statistical analysis

Pearson's chi-square test was used to analyze the distribution of the different yeast species among the collection sites. The exact proportion test, considering a 50% hypothesis, was used to verify differences in yeast recovery during the rainy and the dry season. The antifungal MICs obtained for the different yeast species were compared through ANOVA and *post hoc* Dunnet's test. The Spearman correlation coefficient was used to measure the correlation between the MICs of each tested drug. The Mann-Whitney's non-parametric test was used to compare the antifungal MICs of the strains obtained from different collection sites. P-values lower than 0.05 indicate statistically significant conclusions.

3. Results and Discussion

A total of 46 isolates were obtained, belonging to eight genera and nine species. Of this total, 30 (65.2%) were from surface water samples, 2 *Candida parapsilosis sensu lato*, 2 *Trichomonascus ciferrii* (*Candida ciferrii*), 7 *Meyerozyma guilliermondii* (*Candida*

guilliermondii), 13 *Debaryomyces hansenii* (*Candida famata*), 2 *Cryptococcus laurentii*, 1 *Kodamaea ohmeri*, 1 *Rhodotorula mucilaginosa* and 2 *T. asahii*; and 16 (34.8%) were from sediment samples, 1 *C. tropicalis*, 1 *C. parapsilosis sensu lato*, 3 *T. ciferrii*, 3 *M. guilliermondii*, 5 *D. hansenii*, 1 *C. laurentii* and 2 *T. asahii* (Table 1). When considering the collection sites, point 4 was the one with the highest amount of isolates (n=18, $P < 0.01$), followed by point 2 (n=11), point 1 (n=10) and point 3 (n=7). There were no predominant species among the collection sites. With respect to seasonal variation, 84.8% of the yeasts (39/46) were isolated in the dry season (July to December, $P < 0.01$) and 15.2% (7/46) were isolated in the rainy season, including the three strains of the *Cryptococcus* genus.

In this study, the genus *Candida* showed the highest number of species, similar to what was observed by Medeiros et al. (2008) and Brandão et al., (2010), with the presence of opportunistic pathogens such as *C. tropicalis*, *M. guilliermondii*, *D. hansenii*, *T. ciferrii* and *C. parapsilosis sensu lato*. *D. hansenii* was the most isolated species from both the surface water and sediment (27.7% and 10.6%, respectively), followed by *M. guilliermondii* (14.9% and 6.4%, respectively), together accounting for 59.6% of the isolates found in this study. These two species are isolated from aquatic environments, especially those eutrophized or contaminated with domestic sewage and industrial wastewater (Boguslawska and Dabrowski, 2001; Brilhante et al., 2011; Dynowska, 1997; Medeiros et al., 2008). *D. hansenii*, in particular, besides indicating eutrophication, appears to be closely associated with the removal of these pollutants from contaminated water (Boguslawska and Dabrowski, 2001). In addition, the larger number of isolates obtained during the dry period may be related to the decrease of water volume and consequent concentration of nutrients, which may have favored the growth of yeasts. The

nutritional concentration may also explain the larger number of strains found in point 4, which comprises an area where there is the discharge of domestic sewage (Deak, 2006).

The MIC values obtained from the *in vitro* susceptibility tests of all the isolates are shown in Table II. In relation to *Candida* (*Debaryomyces*, *Meyerozyma*, *Trichomonascus*) species, the MICs for amphotericin B varied from 0.03125 to 2 µg/mL, with two resistant *M. guilliermondii* isolates (MIC = 2 µg/mL), which were also resistant to itraconazole. The MICs for fluconazole ranged from 0.5 to 500 µg/mL and those for itraconazole varied from 0.0625 to 32 µg/mL. A positive correlation was observed between amphotericin B and itraconazole ($P < 0.01$) and fluconazole and itraconazole ($P < 0.01$). Of the 37 *Candida* spp. isolates, seven (18.9%) were resistant to both azole derivatives, five (13.5%) were resistant to itraconazole and one (2.7%) was resistant to fluconazole. Among the azole resistant isolates, three were recovered from point 1 (*D. hansenii*, *M. guilliermondii* and *T. ciferrii*), two from point 2 (*D. hansenii* and *M. guilliermondii*), four from point 3 (2 *D. hansenii*, *C. tropicalis* and *T. ciferrii*) and four from point 4 (3 *M. guilliermondii* and *T. ciferrii*). No statistically significant differences were observed in the antifungal MICs against strains from different collection sites. The MICs for amphotericin B, fluconazole and itraconazole against the other yeast genera varied from 0.0625 to 0.5 µg/mL, 0.25 to 4 µg/mL and 0.03125 to 0.5, respectively (Table II). The antifungal MICs of the *T. asahii* isolates were statistically lower than those of the other yeast genus ($P < 0.05$).

Regarding antifungal resistance to amphotericin B, Savini et al., (2010) reported that *M. guilliermondii* is less susceptible to this drug, when compared to other *Candida* species. The susceptibility of *Candida* isolates to the azole derivatives found in this work corroborates that observed in our previous study using yeasts isolated from wild-harvested freshwater prawns (*Macrobrachium amazonicum*), where a high number of

azole resistant isolates were recovered (Brilhante et al., 2011). In the present study, of the 37 *Candida* spp. isolates, 13 (35.14 %) were resistant to azoles, of which four were *D. hansenii* and five were *M. guilliermondii*. Unlike clinical isolates of *D. hansenii* and *M. guilliermondii*, antifungal resistance has often been described in environmental isolates of these *Candida* species (Brilhante et al., 2011, Medeiros et al., 2008).

Several studies relating the recovery of antifungal resistant environmental yeasts to the degradation of aquatic systems have been published (Brilhante et al., 2011; Medeiros et al., 2008; Brandão et al., 2010). Knowing the mechanisms of antifungal resistance seems like an essential step to better understand the relationship between the development of antifungal resistance and environmental pollution. In order to investigate the mechanisms involved in the azole resistance, the efflux pump inhibition assay was performed with promethazine and haloperidol, which resulted in the reversal of resistance to itraconazole and fluconazole in all tested *Candida* isolates. The addition of promethazine led to an MIC reduction of 8-256-fold and of 8-62.5-fold, for fluconazole and itraconazole, respectively, while the addition of haloperidol led to a 32-125-fold reduction of the MICs for fluconazole (Table 2).

Promethazine is a phenothiazine derivative that acts on MDR and CDR efflux pumps (Kolaczowski et al., 2003), while haloperidol acts only on MDR pumps (Iwaki et al., 2006). Thus, the inhibition of the efflux pump activity by promethazine and haloperidol, resulting in the reversal of the azole resistance, suggests that the azole resistance among the *Candida* spp. strains is related to the enhanced activity of these pumps. This increased activity is a direct result of the upregulation of the CDR and MDR genes, possibly, as a consequence of the presence of different chemical compounds in Catú Lake, secondary to human activities. It is believed that this resistance phenomenon is related to the discharge of industrial wastewater and other pollutants into the aquatic

environment, which may lead to alterations in gene expression or gene sequence in microorganisms (Keenan et al., 2007; Müller et al., 2007; Wegrzyn and Czyz, 2003), and these are the main genetic alterations associated with the development of antifungal resistance (Feng et al., 2010).

The present study reports a high rate of azole resistant *Candida* spp. (*Debaryomyces*, *Meyerozyma*, *Trichomonascus*) strains recovered from an aquatic environment, which may represent a risk for environmental and human health. This article is the first to report the involvement of efflux pumps in the azole resistance among *Candida* spp. from environmental sources. Finally, *D. hansenii* and *M. guilliermondii* were the most commonly isolated species in this study and presented the highest rate of azole resistance. Considering that these species are associated with environmental degradation, these features encourage their use for monitoring the environmental health of water bodies.

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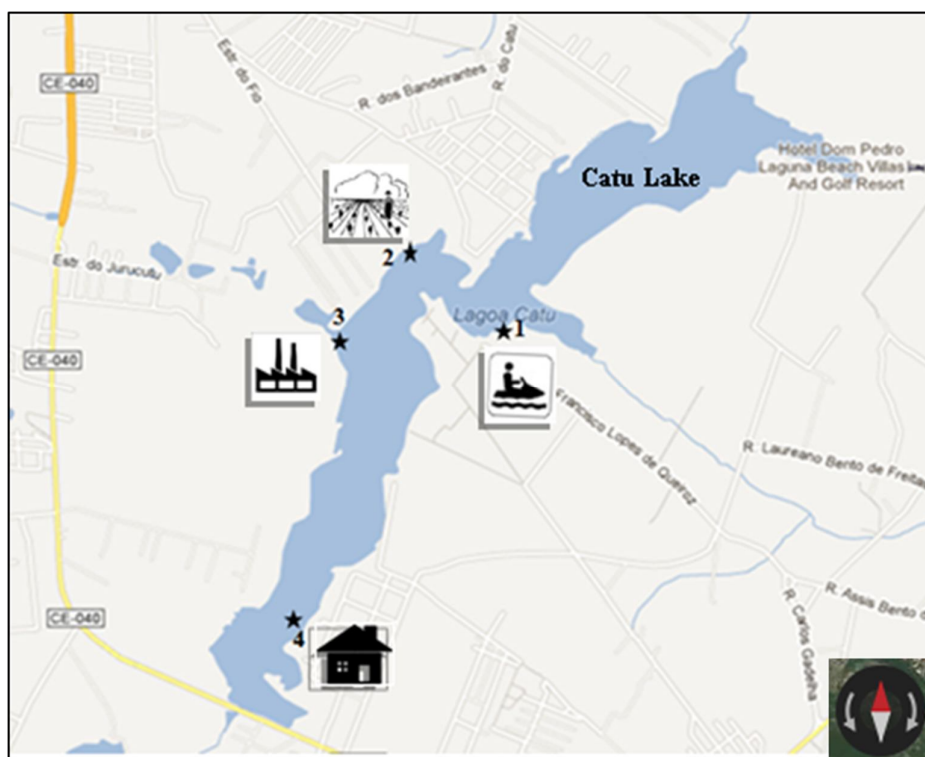


Fig. 1 – Water collection points in Catú Lake, Aquiraz, Ceará, Brazil.

Point 1: Leisure area: bars, restaurants, boats. Area used for activities such as boating and jet skiing. $3^{\circ}55'59.79''$ S and $38^{\circ}21'50.10''$ W; Point 2: Agricultural area, with potato and bean fields, with possible use of azoles. $3^{\circ}55'47.25''$ S and $38^{\circ}22'14.16''$ W; Point 3: Industrial area, near the state highway (CE-040). $3^{\circ}56'03.70''$ S and $38^{\circ}22'25.15''$ W; Point 4: Residential area, discharge of raw household sewage, near the confluence with the Catú River. $3^{\circ}56'56.72''$ S and $38^{\circ}22'31.57''$ W.

1 Table 1: Yeast species isolated from different collection points of Catu Lake.

Yeast Species	Collection Points																	
	Surface Water								Bottom Water								Total	
	Point 1		Point 2		Point 3		Point 4		Point 1		Point 2		Point 3		Point 4		Total	
	n	%	n	%	N	%	N	%	n	%	n	%	n	%	n	%	n	%
<i>Candida tropicalis</i>	-	-	-	-	-	-	-	-	-	-	-	-	1	2.1	-	-	1	2.1
<i>C. parapsilosis</i>	1	2.1	-	-	-	-	1	2.1	-	-	1	2.1	-	-	-	-	3	6.5
<i>C. ciferrii</i> (<i>Trichomonascus ciferrii</i>)	1	2.1	-	-	1	2.1	-	-	1	2.1	-	-	-	-	2	4.3	5	10.8
<i>C. guilliermondii</i> (<i>Meyerozyma guilliermondii</i>)	1	2.1	2	4.3	-	-	4	8.6	-	-	1	2.1	1	2.1	1	2.1	10	21.7
<i>C. famata</i> (<i>Debaryomyces hansenii</i>)	3	6.5	4	8.6	2	4.3	4	8.6	1	2.1	-	-	2	4.3	2	4.3	18	39.1
<i>Cryptococcus laurentii</i>	-	-	-	-	1	2.1	-	-	1	2.1	-	-	-	-	1	2.1	3	6.5
<i>Kodamaea ohmeri</i>	-	-	1	2.1	-	-	-	-	-	-	-	-	-	-	-	-	1	1.8
<i>Rhodotorula mucilaginosa</i>	-	-	1	2.1	-	-	-	-	-	-	-	-	-	-	-	-	1	3.5
<i>Trichosporon asahii</i>	-	-	-	-	-	-	2	4.3	-	-	-	-	-	-	2	4.3	4	8.6
Total	6	13	8	14	4	8.5	11	23.4	4	8.5	2	4.3	4	8.5	8	14	46	100

Table 2: Minimum inhibitory concentration (MIC) of amphotericin B, itraconazole and fluconazole against 46 yeast isolates from Catú Lake.

Species	MIC (µg/mL)					
	n	AMB	FLZ	FLZ + PRO	FLZ + HAL	ITZ ITZ + PRO
<i>C. famata</i> (<i>Debaryomyces hansenii</i>)	18	1 (2) ^a	64 (4)*	4 (1)	2 (2)	4 (2)* 0.125 (1)
		0.5 (4)	32 (4)	2 (3)	1 (2)	2 (1)* 0.0625 (3)
		0.25 (2)	16 (7)			1 (1)*
		0.125 (5)	2 (3)			0.25 (3)
		0.0625 (4)				0.125 (3)
		0.03215 (1)				0.0625 (8)
<i>C. guilliermondii</i> (<i>Meyerozyma guilliermondii</i>)	10	2 (2)	500 (1)*	8 (1)	4 (1)	32 (1)* 0.25 (2)
		1 (3)	64 (1)*	2 (1)	2 (1)	16 (4)* 0.125 (2)
		0.5 (4)	32 (1)			0.25 (1) 0.0625 (1)
		0.125 (1)	16 (1)			0.125 (3)
			4 (1)			0.0625 (1)
			2 (3)			
			1 (2)			
<i>C. parapsilosis</i>	3	1 (3)	2 (1)			0.5 (1) 0.125 (2)
			1 (2)			
<i>C. tropicalis</i>	1	1	250*	8	2	32* 0.25
<i>C. ciferrii</i> (<i>Trichomonascus ciferrii</i>)	5	1 (2)	64 (1)*	8	2	16 (1)* 0.125 (2)
		0.5 (2)	32 (1)			1 (1)*
		0.125 (2)	4 (1)			0.125 (2)
			2 (1)			0.0625 (1)
			0.5 (1)			
<i>Kodamaea ohmeri</i>	1	0.25	0.5			0.125
<i>Cryptococcus laurentii</i>	3	0.5 (3)	2 (2)			0.125 (1)
			4 (1)			0.5 (2)
<i>Rhodotorula mucilaginosa</i>	1	0.0625	0.25			0.03125
<i>Trichosporon asahii</i>	4	0.125 (1)	0.5 (2)			0.125 (1)
		0.0625 (3)	0.25 (2)			0.0625 (2)
						0.03125 (1)

^a Represents the number of isolates for each MIC indicated. AMB: amphotericin B, FLZ: fluconazole;

ITZ: itraconazole; PRO: promethazine; HAL: haloperidol.

* Strains tested with efflux pump inhibitors

10 CONCLUSÃO

1 A principal espécie de *Candida* componente da microbiota por leveduras de *M. amazonicum* foi *C. famata*, seguido por *C. guilliermondii*.

2 As principais espécies de *Candida* presentes na água da lagoa do Catu, Aquiraz, Ceará-Brasil foram *C. famata* e *C. guilliermondii*.

3 O fenômeno de resistência perante o itraconazol e o fluconazol está presente em *Candida* spp. isoladas de *M. amazonicum* e do ambiente, mas não está presente em cepas dos gêneros *Rhodotorula*, *Cryptococcus*, *Trichosporon* e *Kodamaea*.

4 As espécies de leveduras *C. famata* e *C. guilliermondii* foram as que exibiram a maior taxa de resistência aos derivados azólicos itraconazol e fluconazol.

5 Os moduladores de atividade de bomba de efluxo Prometazina (Fenotiazina) e Haloperidol (Butirofenona) foram eficientes para detecção de resistência a azólicos mediada por bombas de efluxo, pois mostraram resultados de redução da concentração inibitória mínima nas cepas resistentes de oito a 256 vezes em relação à inicial.

6 Foi observado 100% de similaridade em espécies de *Candida* isoladas de camarões e do ambiente aquático, demonstrando que o uso de *M. amazonicum* como sentinela para o isolamento de *Candida* spp. em ambientes de água doce é uma alternativa para a avaliação da qualidade ambiental.

11 PERSPECTIVAS

Este trabalho é o primeiro a relatar bombas de efluxo envolvidas na resistência aos derivados azólicos em cepas de *Candida* spp. de ambientes aquáticos dulcícolas. Considerando que *C. famata* e *C. guilliermondii* são associadas com a degradação ambiental, e que foram as espécies mais isoladas nesse estudo, apresentando a maior taxa de resistência aos azólicos, o uso destas espécies para o monitoramento da saúde ambiental dos corpos d'água doce parece promissor.

Adicionalmente, com base nos resultados, o uso de *M. amazonicum* como sentinela para o isolamento de *Candida* spp. a partir de ambientes aquáticos dulcícolas é uma alternativa interessante para a avaliação da qualidade desses ambientes, considerando-se que esses animais abrigam leveduras do ambiente no seu trato gastrointestinal, com um maior número de isolados quando comparado ao isolamento direto desse tipo de ambiente. Além disso, em razão da sua capacidade de acumular poluentes químicos nos seus tecidos, simulam as condições ambientais em que essas leveduras estão expostas, o que pode contribuir para o controle da presença de resistentes à *Candida* spp. nestes ambientes.

Por fim, o elevado número de isolados de *Candida* spp. resistentes aos antifúngicos fluconazol e itraconazol, bem como o isolamento de cepas de *Candida guilliermondii* resistentes ao antifúngico amfotericina B, podem representar um risco para a saúde ambiental e humana.

Assim, mais estudos devem ser conduzidos na tentativa de entender a relação entre o desenvolvimento de resistência aos derivados azólicos e a presença de poluentes em ecossistemas aquáticos. Ante o exposto, essa ferramenta se torna promissora para o monitoramento ambiental, contudo, ainda não se sabe o quão sensíveis esses microrganismos são aos vários poluentes, não se conhecendo, portanto, a quantidade de poluente necessária para provocar alterações fenotípicas nessas leveduras.

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