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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

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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 25ºC, 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, ≥1µg 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/ HCI (pH 8.3), 50mM of KCl, 1.5mM of MgCl2, 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 10µL, 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.

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

The recovered ***Candida*** species were simultaneously isolated from prawns and the aquatic environment and some of these isolates presented 100% 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% 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.

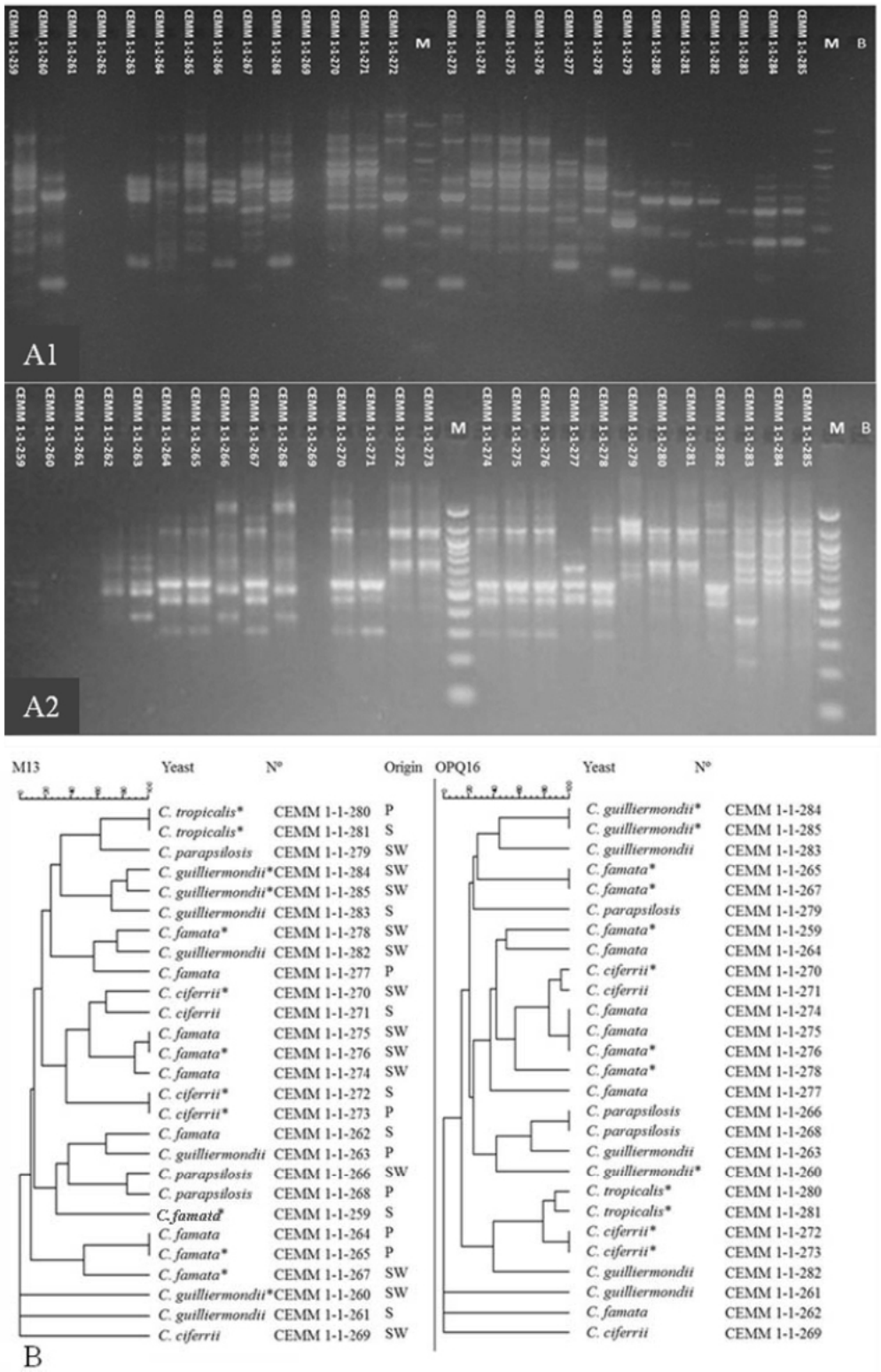

Figure 1 - Dendrograms resulting from the analysis of 27 isolates of ***Candida*** spp. obtained from the gastrointestinal tract of ***Macrobrachium amazonicum*** (n=7) and the natural environment (n=20) through M-13-fingerprinting and RAPD-PCR with primer OPQ16. P: prawn; SW: surface water; S: sediment.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

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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REFERENCES

AMERICAN PUBLIC HEALTH ASSOCIATION (APHA); AMERICAN WATER WORKS ASSOCIATION (AWWA); WATER ENVIRONMENTAL FEDERATION (WEF). **Standard methods for the examination of water and wastewater**. 20.ed. Washington, 1998. 64p.

BRILHANTE, R.S.N. et al. Yeast microbiota of raptors: a possible tool for environmental monitoring. **Environmental Microbiology Reports**, v.4, n.2, p.189-193, 2012. Available from: <http://onlinelibrary.wiley.com/doi/10.1111/j.1758-2229.2011.00319.x/abstract>. Accessed: Nov. 08, 2013. doi: 10.1111/j.1758-2229.2011.00319.x.

BRILHANTE, R.S.N. et al. Yeasts from ***Macrobrachium amazonicum***: a focus on antifungal susceptibility and virulence factors of ***Candida*** spp. **FEMS Microbiology Ecology**, v.76, n.2, p.268-277, 2011. Available from: <http://onlinelibrary.wiley.com/doi/10.1111/j.1574-6941.2011.01050.x/abstract>. Accessed: Nov. 08, 2013. doi: 10.1111/j.1574-6941.2011.01050.x.

BUTINAR, L. et al. Yeast diversity in hypersaline habitats. **FEMS Microbiology Letters**, v.15, n.2, p.229-234, 2005. Available from: <http://onlinelibrary.wiley.com/doi/10.1016/j.femsle.2005.01.043/pdf>. Accessed: Nov. 08, 2013. doi:10.1016/j.femsle.2005.01.043.

CASTELO-BRANCO, D.S.C.M. et al. Azole-resistant ***Candida albicans*** from a wild Brazilian porcupine (***Coendou prehensilis***): a sign of an environmental imbalance? **Medical Mycology**, v.51, n.5, p.555-560, 2013. Available from: <http://informahealthcare.com/doi/abs/10.3109/13693786.2012.752878>. Accessed: Nov. 08, 2013. doi: 10.3109/13693786.2012.752878.

CLINICAL AND LABORATORY STANDARDS INSTITUTE. **Reference method for broth dilution antifungal susceptibility testing of yeast**: approved guideline M27-A3. Wayne, PA: CLSI, 2008. V.28, p.1-25.

DE HOOG, G.S. et al. **Atlas of clinical fungi**. 2.ed. The Nederlands: Centraalbureau voor Schimmelcultures, Baarn, 2000. 1126 p.

FENG, L. et al. Relationship between antifungal resistance of fluconazole resistant ***Candida albicans*** and mutations in ERG11 gene. **Chinese Medical Journal**, v.123, n.5, p.544-548, 2010. Available from: <http://www.cmj.org/ch/reader/view_abstract_ext.aspx?file_no=20103842232480&flag=1>. Accessed: Dec. 20, 2013. doi: 10.3760/cma.j.issn.0366-6999.2010.05.007.

JUNGWIRTH, H.; KUCHLER, K. Yeast ABC transporters – a tale of sex, stress, drugs and aging. **FEBS Letters**, v.580, n.4, p.1131-1138, 2006. Available from: <http://www.sciencedirect.com/science/article/pii/S0014579305015334>. Accessed: Dec. 20, 2013. doi:10.1016/j.febslet.2005.12.050.

KEENAN, P.O. et al. Clear and present danger? The use of a yeast biosensor to monitor changes in the toxicity of industrial effluents subjected to oxidative colour removal treatments. **Journal of Environmental Monitoring**, v.9, n.12, p.1394-1401, 2007. Available from: <http://pubs.rsc.org/en/Content/ArticleLanding/2007/EM/b710406e#!divAbstract>. Accessed: Nov. 08, 2013. doi**:** 10.1039/B710406E.

MEDEIROS, A.O. et al. Diversity and antifungal susceptibility of yeasts from tropical freshwater environments in Southeastern Brazil. **Water Research**, v.42, n.14, p.3921-3929, 2008. Disponível em: <http://www.sciencedirect.com/science/article/pii/S0043135408002522>. Accessed: Nov. 08, 2013. doi: 10.1016/j.watres.2008.05.026.

MÜLLER, F.M.C. et al. Cross-resistance to medical and agricultural azole drugs in yeasts from the oropharynx of human immunodeficiency virus patients and from environmental bavarian vine grapes. **Antimicrobial Agents and Chemotherapy**, v.51, n.8, p.3014-3016, 2007. Available from: <http://aac.asm.org/content/51/8/3014.long>. Accessed: Nov. 08 2013. doi:10.1128/AAC.00459-07.

VIRGA, R.H.P. et al. Assessment of heavy metal contamination in blue crab specimes. **Food Science and Technology**, v.27, n.4, p.779-785, 2007. Available from: <http://www.scielo.br/scielo.php?pid=S0101-20612007000400017&script=sci_arttext>. Accessed: Nov. 08, 2013. doi: 10.1590/S0101-20612007000400017.

YILMAZ, A.B.; YILMAZ, L. Influences of sex and seasons on levels of heavy metals in tissues of green tiger shrimp (***Penaeus semisulcatus*** de Hann, 1844). **Food Chemistry**, v.101, n.4, p.1664-1669, 2007. Available from: <http://www.sciencedirect.com/science/article/pii/S0308814606003438>. Accessed: Nov. 08, 2013. doi: 10.1016/j.foodchem.2006.04.025.