PHENOPTYPIC SWITCHING ALTERS VIRULENCE AND FLUCONAZOLE SUSCEPTIBILITY PROFILE IN Candida tropicalis CLINICAL ISOLATE

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ABSTRACT

Phenotypic switching provides morphological alterations in colonies of Candida tropicalis resulting in cellular and metabolic changes in this yeast. This species has increased incidence rates in the last decades with prevalence in tropical regions. C. tropicalis exhibits different virulence factors, including the phenotypic switching, that can change others virulence traits and generate more adapted strains. In this study, we evaluated the effect of switching event on virulence characteristics and fluconazole susceptibility using a switch variant strain derived from a clinical isolate of C. tropicalis. We observed reduction in phagocytosis rates by hemocytes infected with the phenotypic variant compared to that observed for the clinical isolate. The phenotypic variant exhibited high capacity of filamentation during infection in G. mellonella. Furthermore, the morphological variant showed an increased expression of the transcription factor EFG1, associated with cellular differentiation, in comparison to the clinical isolate. The switching was also related to changes in minimum inhibitory concentration (MIC) to fluconazole. MIC of the variant was 256 times higher than the MIC of the clinical isolate. Our results demonstrate that the switching changes the profiles of virulence and susceptibility to fluconazole, resulting in a variant strain potentially more pathogenic.

KEYWORDS: Candida tropicalis, phenotypic switching, Galleria mellonella, filamentation, fluconazole susceptibility.

1. INTRODUCTION

Invasive fungal diseases are a life-threatening problem for immunocompromised patients and are frequently caused by Candida species1. Candidemia episodes have been considerably rising in the last decades and remain an important comorbid condition in hospitals2,3.

The epidemiology of candidemia is generally characterized by geographical and temporal variability. Historically, C. albicans has been the most common isolated species from candidemia; however, there has been a reported increase in the incidence of non-C. albicans species4,5. C. tropicalis is frequently isolated from patients with candidiasis, being often described as the first or second non-C. albicans species involved in cases of candidemia and candiduria in Brazil6,7.

However, in some cases C. tropicalis exceeds C. albicans in frequency of isolation, configuring the importance of this pathogen in the Brazilian clinical scenario8,9. The Galleria mellonella alternative model has been demonstrate high efficiency in the investigation of microbial pathogens, include Candida species10,11,12. This model presents advantages compared to other invertebrates and mammalian models, like can be maintained between 25 °C and 37 °C, the inoculum can be injected direct in hemolymph and the cultivation costs are less expensive than the murine models13,10. G. mellonella larvae presents sensitive to infection with phenotypic switching morphotypes of C. tropicalis, however little is known about the relationship of this pathogen and the immune system of the invertebrate host12.

To ensure infection success and pathogenicity manifestation C. tropicalis needs to bring up different virulence factors14,15,16. Phenotypic switching promotes variability in isogenic populations, reversible changes in macro-morphology of colonies and differentiated virulence profiles. Phenotypic switching can cause changes in transcriptional level (epigenetic), besides macroscopic changes, can also lead cellular alterations, as well as, metabolic and signaling pathways, culminating in alteration of virulence network in C. tropicalis, generating individuals that, although sensitive to infection with C. albicans, can be resistant to antifungal agents in regiments of prophylaxis and empirical therapy, resulting in the emergence of resistant clinical isolates, particularly against triazoles and echinocandins20,21.

In this scenario, the recent emergence of fluconazole resistance among isolates of species that are usually primarily sensitive to this drug like C.
trópicales revela a importância de estudos sobre essas características. Azóis são amplamente utilizados para controlar infecções por fungos, nos últimos anos, tem sido relatado aumento na resistência a esses fungos em C. tropicalis, resultando em infecções persistentes que aumentam a morbidade e mortalidade, especialmente em pacientes imunocomprometidos.

Em nosso estudo, um isolado do C. tropicalis e seus derivados morfotipos variados foram usados para identificar o potencial de células filamentosas com alterações da virulência e susceptibilidade ao fluconazol.

**2. MÉTODOS**

**Microorganismos e isolados fúngicos**

Neste trabalho, utilizamos a cepa clínica 49.007 de C. tropicalis que exibe um padrão de colonização estándar (Nomeado Morfotipo) e um derivado variante com um padrão colonização estruturado (nomeado morfotipo de crepe). Os morfotipos foram armazenados em estoques congelados a 20% glicerol a -80°C e cultivados em placas de YPD agar para (1% Yeast Extract, 2% Peptone, 2% Glucose e 2% Ágar) a 28°C. As bordas foram estabelecidas em YPD líquido a 28°C, em uma incubadora a 28°C.

**In vivo phagocytosis assay**

O ensaio de fagocitose in vivo foi realizado seguindo Scorzon et al. (2013) com modificações. As células foram preparadas em meio PBS e ajustadas para 1.0×10⁶ células/mL. Suspendam-se em meio Calcofluor White (10 mg/mL) e incubadas por 30 min a 37°C. A suspenção foi transferida em larvas de Galleria mellonella não parasitadas. O inseto foi incubado em ambiente controlado de temperatura (37°C) por 6 horas, mantendo a proteção do ambiente. Após a incubação, as hemólidas foram coletadas em eau de mercurio (EDTA) anticoagulante. As células filamentosas foram contadas no hemocitómetro e a porcentagem de fagocitose foi calculada ao longo do tempo, comparando a cepa clínica e o padrão virulento.

**EFG1 Gene expression**

As larvas foram inoculadas como descrito acima. Em média, 1 h e 4 h após a infecção, três larvas de cada grupo foram inoculadas em meio YPD e cultivadas a 28°C. Após 96 horas de incubação, as cepas fúngicas foram isoladas e cultivadas em YPD agar. O isolate de C. tropicalis 49.007 e os morfotipos de crepe foram testados em meio de hemólidas de Galleria mellonella. As cepas foram subcultivadas em meio YPD agar e armazenadas em temperaturas baixas para conservação.

**Testing fluconazole susceptibility**

O método de testagem de susceptibilidade ao fluconazol foi realizado seguindo os critérios do CLSI. As cepas foram inoculadas em meio YPD e cultivadas a 37°C por 24 horas. Após a incubação, o meio foi transferido em hemólidas de Galleria mellonella não parasitadas. O inseto foi incubado em ambiente controlado de temperatura (37°C) por 6 horas, mantendo a proteção do ambiente. Após a incubação, as hemólidas foram coletadas em eau de mercurio (EDTA) anticoagulante. As células filamentosas foram contadas no hemocitómetro e a porcentagem de fagocitose foi calculada ao longo do tempo, comparando a cepa clínica e o padrão virulento.

**Statistical analysis**

As análises estatísticas foram realizadas através do teste t não-paramétrico de Mann-Whitney. As diferenças foram consideradas significantes quando p < 0,05. O software utilizado foi o GraphPad Prism (GraphPad Software, La Jolla, USA, versão 6.01).

**3. RESULTS**

In vivo phagocytosis assay

Depois da incubação, G. mellonella mostrou capacidade de fagocitose das duas cepas. 20% de hemólidas de larvas infectadas com cepa clínica mostraram internalização de células filamentosas. A cepa clínica induziu redução na taxa de fagocitose (5%) (Figura 1).
Capacity of morphogenesis in vivo

Morphotypes showed differences in the capacity of morphogenesis (transition between yeast forms and filamentous forms) during infection. Phenotypic variant exhibited higher capacity of filamentation, presenting 10% of total cells observed in the hemolymph of *G. mellonella* in the hyphal stage. Clinical isolate (parental morphotype) showed lower capacity of morphological transitions (6%) (Figure 1).

Figure 1. *In vivo* phagocytosis of *Galleria mellonella* hemocytes in clinical isolate and switched morphological variant after 6 h of infection (A). The results are shown as mean ± SD. Mann-Whitney test. *p < 0.05. Hemocytes presenting yeast cells internalized of clinical isolate (B) and morphological variant (C). The capacity of morphogenesis of clinical isolate and morphological variant post-infection in hemolymph of *Galleria mellonella* larvae (D). The results are shown as mean ± SD. Unpaired t-test. *p < 0.05. Yeast forms presented of clinical isolate (E) and filamentous forms presented by morphological variant (F).

**EFG1 Gene expression**

The morphological variant showed increased expression of the transcription factor *EFG1* after 1 hour post-infection in comparison to the expression of the clinical isolate. After 4 hours post-infection, the expression of *EFG1* was even higher (about twice) by the switched strain in relation to the expression of the clinical isolate (Figure 2).

**Testing fluconazole susceptibility**

The MIC values of fluconazole differed considerably between the clinical isolate of *C. tropicalis* (MIC ≤ 0.125 μg/ml) and its phenotypic variant (MIC = 32 μg/ml) (Table 1). This data indicates that this altered susceptibility is due to the switched state of the variant.

**Figure 2.** Relative *EFG1* gene expression of clinical isolate of *Candida tropicalis* and its switched morphological variant after 1 h and 4 h of infection in *Galleria mellonella* larvae. Data was normalized with β-actin gene and relativized with the expression of clinical isolate. The results are shown as mean ± SD. Unpaired t-test. *p < 0.05.

**Table 1.** MIC of fluconazole for 49.07 isolate (clinical isolate and variant) of *Candida tropicalis*.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Fluconazole MIC (μg/ml)</th>
<th>Breakpoints CLSI-2008</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>S (≤8 μg/ml)</td>
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<tr>
<td>Clinical isolate</td>
<td>32</td>
<td>S</td>
</tr>
<tr>
<td>Crepe</td>
<td>0.125</td>
<td>S</td>
</tr>
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S-susceptible; SDD-susceptible-dose dependent.

4. **DISCUSSION**

*G. mellonella* exhibits an innate immune system similar to innate system presented in mammalian. The use of *G. mellonella* larvae to study the pathogenicity of various microbial pathogens, including phenotypic switching morphotypes of *C. tropicalis*, has been reported recently. Differences in mortality in this...
larvae caused by phenotypic switching morphotypes of the clinical isolate 49.07 of \textit{C. tropicalis} have been demonstrated\textsuperscript{12} and differences in the hemolytic capacity and changes in susceptibility to itraconazole have also been verified\textsuperscript{19}.

In the present work we demonstrated reduction in the capacity of phagocytosis by hemocyte of \textit{G. mellonella} larvae mediated by the phenotypic switching event. The phagocytic cells in insects, have receptors on the surface which are similar to receptors on mammalian neutrophils\textsuperscript{31,32}, both cells engulf and kill pathogens and produce superoxide using similar pathways\textsuperscript{32}. This event suggests possible similar responses to the presence of pathogens and morphological changes modifying recognition profiles in \textit{C. tropicalis} may be of great clinical relevance.

The increased of phagocytosis can be induced by the higher capacity of filamentation of switched morphotype presented here. This event can also be observed in the increase of the \textit{EFG1} expression. This transcription factor regulates filamentation and biofilm formation in \textit{C. tropicalis}. These data suggest that it gene can be integrate a regulatory network of phenotypic switching event in \textit{C. tropicalis}, which may alter virulence profiles and other factors related to the microevolution event. Morphological transitions are related with pathogenicity of \textit{C. tropicalis}\textsuperscript{33}. The mechanism of morphogenesis contributes to virulence include evasion of phagocytosis\textsuperscript{34,30} since the immune system responds differently to yeasts cells and filamentous forms\textsuperscript{35,30}. Mesa-Arango \textit{et al} (2013)\textsuperscript{36} observed filamentous forms of \textit{C. tropicalis} inside the hemocytes, suggesting that the ability to induce filamentation after phagocytosis provide a mechanism to escape form phagocytic cells. These authors also noted that individuals who presented greater filamentation caused bigger decline in the number of hemocytes, resulting in lower capacity of phagocytosis and consequent increase in virulence. In this scenario, a phenotypic variant provides growth versatility in metabolic and cellular responses resulting in a higher infection success\textsuperscript{36}.

We also investigated the effect of the switching on fluconazole susceptibility. The results showed a direct relation of this event with increase of resistance for this azole, increasing in 256 times the MIC of the morphological variant in contrast to the clinical isolate (parental morphology). Moralez \textit{et al} (2014)\textsuperscript{19} observed that phenotypic switching also altered the MIC for itraconazole in \textit{C. tropicalis}.

The decrease in susceptibility to drugs used to control infections it is considered a microevolution\textsuperscript{37}, which may be increased by the phenotypic switching event\textsuperscript{9,38}. This micro evolutionary context brings concerns to the clinical environment, since infections caused by \textit{C. tropicalis} resistant to azoles are emerging in the last years\textsuperscript{27,9}.

5. CONCLUSION

This work can conclude that the event of phenotypic switching interferes on virulence potential by altering cellular characteristics in \textit{C. tropicalis}. These alterations bring up the morphotype variant more virulent compared to the clinical isolate. Thus \textit{C. tropicalis} strains that present morphological changes caused by phenotypic switching may represent an important problem in clinical context, resulting in difficult treatment and increasing hospitalization time, even decreasing the patient's survival. The knowledge of the regulatory network of this epigenetic event may help to the development of strategies to prevent \textit{C. tropicalis} infections.

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Correlates with the yeast in...–ive; and, breaking through a common host pathogen.

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