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

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Received: 05/16/2018; **Accepted:** 06/07/2018

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 generates 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 phagocytic 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 *EFG*1, 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* species[1]. Candidemia episodes have been considerably rising in the last decades and remain an important comorbid condition in hospitals[2,3].

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* species[4,5]. *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 Brazil[6,7]. However in some cases *C. tropicalis* exceeds *C. albicans* in frequency of isolation, configuring the importance of this pathogen in the Brazilian clinical scenario[8,9].

The *Galleria mellonella* alternative model have been demonstrate high efficiency in the investigation of microbial pathogens, include *Candida* species[10,11,12]. 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 models[13,10]. *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 host[12].

To ensure infection success and pathogenicity manifestation *C. tropicalis* needs to bring up different virulence factors[14,15,16]. 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 isogenic express distinct virulence profiles, and antifungal susceptibility patterns[17,18,19]. Recognition of the importance of *Candida* infections has led to a significant increase in the use of antifungal agents in regiments of prophylaxis and empirical therapy, resulting in the emergence of resistant clinical isolates, particularly against triazoles and echinocandins[20,21].

In this scenario, the recent emergence of fluconazole resistance among isolates of species that are usually primarily sensitive to this drug like *C.*

tropicalis reveals the importance of studies on these characteristics[22,23,24]. Azoles are widely used to control yeast infections, however in the last decade azole resistence have been frequently reported in clinical isolates of *C. tropicalis*[25,26], resulting in persistent infections, increasing the morbidity and mortality rates in immuno-compromised patients[27].

In this study, a clinical isolate of *C. tropicalis* and its derived phenotypic switching variant were used in order to evaluate the potential raise of strains with altered virulence traits and susceptibility to fluconazole.

2. METHODS

Microbial strains and Fungal inocula preparation

In this work we used the clinical strain 49.07 of *C. tropicalis* that exhibits a smooth standard colony pattern (named smooth morphotype) and a derived variant strain with a more structured colony pattern (named crepe morphotype). The morphotypes were stored as frozen stocks with 20% glycerol at -80°C and subcultured on YPD agar plates (1 % Yeast Extract, 2 % Peptone, 2 % Glucose and 2 % Ágar) at 28°C. Strains were routinely grown in YPD liquid medium at 28°C in a shaking incubator and plated in YPD agar plates at 28°C for 96 hours.

In vivo phagocytosis assay

In vivo phagocytosis assay was performed according Scorzoni *et al.* (2013)[28] with modifications. Cells of morphotypes (clinical strain and switched crepe variant) were suspended in PBS and adjusted for $1x10^8$ cells/mL. Suspensions were stained with Calcofluor white (10 µg/mL) and incubated for 30 min at 37 °C. 10 µl of these suspensions was injected in hemocell of larvae of *Galleria mellonella* through the last left pro-leg. The insect was incubated for 6 hours under protection from light at 37 °C. After incubation hemolymph was collected in IPS buffer (insect physiological saline: 150 mM sodium chloride, 5 mM potassium chloride, 10 mM Tris HCl pH 6.9, 10 mM EDTA and 30 mM sodium citrate) plus 10 mM N-ethylmaleimide (anticoagulant). Hemocyte were applied in hemocytometer and counted in fluorescent microscopy. Percentage of phagocyted cells was determined by the ratio between hemocyte containing yeast cells and total hemocyte number.

Capacity of morphogenesis in vivo

G. mellonella larvae were injected with morphotypes as described above following incubation at 37 °C for 24 hours. After incubation, hemolymph was collected in IPS plus anticoagulant and concentrated twice. Number of the filamentous cells was counted in hemocytometer and percentage of filamentation capacity was determined by the ratio of the filamentous cells and the total number of yeasts cells.

*EFG*1 Gene expression

Larvae were inoculated as described above. At both 1 h and 4 h post infection, 3 larvae from each experimental group were snap-frozen in liquid nitrogen and ground to a powder by mortar and pestle in TRIzol. The samples were further homogenized, and RNA was extracted and purified using PureLink® RNA Mini Kit according manufacturer's instructions. RNA was quantified and quality assessed using a NanoDrop spectrophotometer. cDNA was synthesized from 200 ng of extracted RNA using a RT-PCR kit in a GeneAmp® PCR following the manufacturer's instructions. Primers used for quantitative PCR (qPCR) for *EFG*1 gene were 5'(TTCAACTGCTGGACAACCAC) and 3'(TACCAGGAGGTTGGAATTGG) and the housekeeping ACT1 gene were 5'(GACCCATCCCCCTTTTTATG) and 3'(TCGATCTTAATCGGGAGGTG). The cycling conditions consisted of 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 51°C and 60 s at 60°C. Each sample was analyzed in duplicate using an StepOnePlus™ Real-Time PCR System. Reaction was performed using Platinum® SYBR® Green qPCR Supermix-UDG with final volume of 20 µl. Relative gene expression was calculated using the $2^{-(\Delta-\Delta Ct)}$ method.

Testing fluconazole susceptibility

The minimum inhibitory concentration (MIC) of fluconazole for a clinical isolate of *C. tropicalis* (49.07) and its phenotypic variant (crepe) was determined according CLSI document M27-A2[29]. The MIC (µg/ml) was established when the MIC value scale for 80% inhibition of growth was reach. MIC interpretations follow the CLSI breakpoints for fluconazol (≤8 ug/ml, S-susceptible; 16-32 ug/ml, SDD-susceptible-dose dependent, ≥64, resistant)[29]. A quality control test was performed employing the standard strain *C. parapsilosis* ATCC 22019.

Statistical analysis

Unpaired t-test or the non-parametric Mann-Whitney test was used depending on the data distribution. Differences were considered significant when $p < 0.05$. Statistical analysis was performed using GraphPad Prism (GraphPad Software, La jolla, USA, version 6.01).

3. RESULTS

In vivo phagocytosis assay

After incubation, *G. mellonella* showed capacity to phagocyte cells of the two morphotypes analyzed. 20% of hemocytes from larvae infected with original morphotype (clinical isolate) showed internalized yeasts. The crepe morphotype (phenotypic variant) induced lower phagocytic rates (5%) (Figure 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).

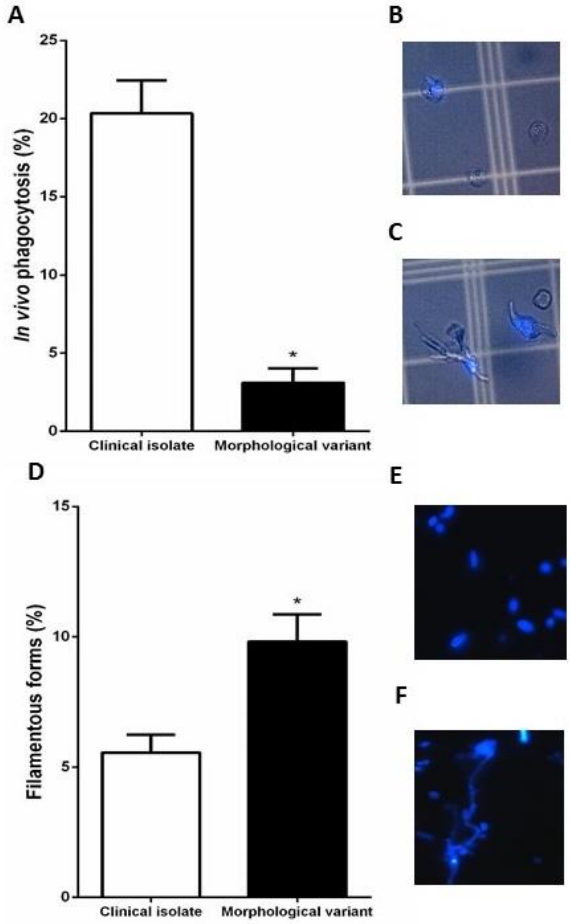

Figure 1. *In vivo* phagocytosis of *Galleria mellonella* hemocytes in clinical isolate and switchted morphological variant after 6 h of infection (A). The results are show as mean ± SD. Mann-Whitney test. *p < 0.05. Hemocytes presenting yeast cells internalized of clinical isolate (B) and morphological 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 *EFG*1 after 1 hour post-infection in comparison to the expression of the clinical isolate. After 4 hours post-infection, the expression of *EFG*1was 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.

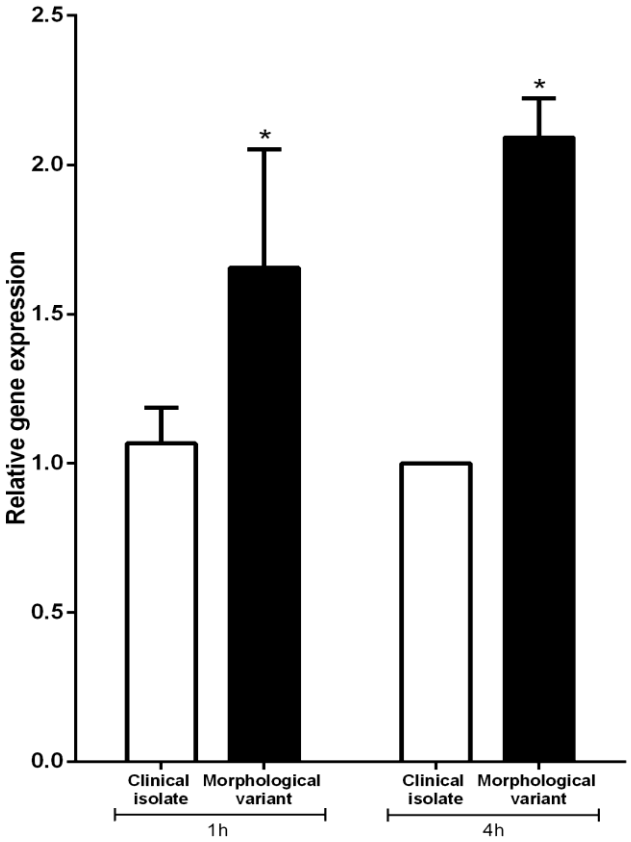

Figure 2. Relative *EFG*1 gene expression of clinical isolate of *Candida tropicalis* and its switchted morphological variant after 1 h and 4 h of infection in *Galleria mellonella* larvae. Data was normalized with β-actin gene and relativized with the expression of clinical isolate. The results are 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.*

		Fluconazole MIC (μg/ml)	*Breakpoints CLSI-2008*
Isolate			
49.07	Clinical isolate	32	≤8 ug/ml - S
	Crepe	0.125	16-32 ug/ml - SDD

S-susceptible; SDD-susceptible-dose dependent.

4. DISCUSSION

G. mellonella exhibits an innate immune system similar to innate system presented in mammalian[30]. 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[12]. Differences in mortality in this

larvae caused by phenotypic switching morphotypes of the clinical isolate 49.07 of *C. tropicalis* have been demonstrated[12] and differences in the hemolytic capacity and changes in susceptibility to itraconazole have also been verified[19].

In the present work we demonstrated reduction in the capacity of phagocytosis by hemocyte of *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[31,32], both cells engulf and kill pathogens and produce superoxide using similar pathways[32]. This event suggests possible similar responses to the presence of pathogens and morphological changes modifying recognition profiles in *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 *EFG*1 expression. This transcription factor regulates filamentation and biofilm formation in *C. tropicalis*. These data suggest that it gene can be integrate a regulatory network of phenotypic switching event in *C. tropicalis*, which may alter virulence profiles and other factors related to the microevolution event. Morphological transitions are related with pathogenicity of *C. tropicalis*[33]. The mechanism of morphogenesis contributes to virulence include evasion of phagocytosis[34,30] since the immune system responds differently to yeasts cells and filamentous forms[35,30]. Mesa-Arango *et al* (2013)[36] observed filamentous forms of *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[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 *et al* (2014)[19] observed that phenotypic switching also altered the MIC for itraconazole in *C. tropicalis*.

The decrease in susceptibility to drugs used to control infections it is considered a microevolution[37], which may be increased by the phenotypic switching event[19,38]. This micro evolutionary context brings concerns to the clinical environment, since infections caused by *C. tropicalis* resistant to azoles are emerging in the last years[27,9].

5. CONCLUSION

This work can conclude that the event of phenotypic switching interferes on virulence potential by altering cellular characteristics in *C. tropicalis*. These alterations bring up the morphotype variant more virulent compared to the clinical isolate. Thus *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 *C. tropicalis* infections.

ACKNOWLEDGMENTS

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) – Brazil; Fundação Araucária/Governo do Paraná – Brazil; Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) – Brazil and PROPPG/UEL – Brazil.

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