

Lack of effect of cell-wall targeted antibacterials on biofilm formation and antifungal susceptibility of *Candida* species

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The use of central venous catheters (CVC) and broad-spectrum antibacterials are among the main risk factors for the development of candidemia in patients admitted to intensive care units (ICU). It is known that some antibacterials increase the resistance of these yeasts to azole antifungals. Thus, the aim of this research was to determine whether yeast present in CVC colonizations previously exposed to cell-wall targeted antibacterials benefit from a reduction in susceptibility to fluconazole and voriconazole, facilitating their ability to form biofilms. *Candida albicans*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis* and *C. guilhermondii* were seeded into antibacterial (cefepime, meropenem, vancomycin, and piperacillin-tazobactam) gradient plates produced in Mueller-Hinton Agar. The susceptibility to fluconazole and voriconazole and the biofilm formation of the yeasts were tested before and after exposure to the antibacterials. None of the antibacterials exerted a significant effect on the *in vitro* susceptibility of the yeasts to the antifungal agents or on their ability to form biofilms. These results suggest that increased candidemia in ICU patients is not attributable to possible alterations in the yeasts, but is more likely caused by a weakening of the patient's general condition after long exposure to infection.

Uniterms: Central venous cateter/use. Candidemia/control. Biofilm/formation. Antibacterials. Antifungals. *Candida*/species/control..

O uso de cateter venoso central (CVC) e antibióticos de amplo espectro estão entre os principais fatores de risco para o desenvolvimento da candidemia em pacientes internados em unidades de terapia intensiva (UTI). É conhecido que alguns antibióticos aumentam a resistência das leveduras aos antifúngicos azólicos. Assim, o objetivo deste estudo foi avaliar se leveduras presentes em colonização de CVC expostas a antibióticos que atuam em parede celular se beneficiam de uma redução na suscetibilidade ao fluconazol e voriconazol e se obtêm maior capacidade de formar biofilme. *Candida albicans*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis* e *C. guilhermondii* foram semeadas em placas de Mueller-Hinton Agar, com gradientes de antibióticos (cefepima, meropenem, vancomicina e piperacilina-tazobactam). A suscetibilidade e a formação de biofilme das leveduras foram testadas antes e após a exposição aos antibióticos. Nenhum dos antibióticos provocou alterações detectáveis *in vitro* sobre a suscetibilidade das leveduras aos antifúngicos ou à capacidade de formar biofilme. Estes resultados sugerem que o aumento da candidemia na UTI não seria atribuído a possíveis alterações provocadas nas leveduras e sim pelo enfraquecimento da condição geral do paciente após longa exposição à infecção.

Unitermos: Cateter venoso central/uso. Candidemia/control. Biofilme/formação. Antibióticos. Antifúngicos. *Candida*/espécies/control.

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INTRODUCTION

The incidence of candidemia in tertiary public hospitals in Brazil is approximately 2.5 cases per 1000 hospital admissions (Colombo *et al.*, 2013). *Candida* species are the fourth-most-common etiological agent of nosocomial bloodstream infections (BSIs), and have a mortality rate of over 40%. Risk-factors for invasive candidiasis include the use of central venous catheters (CVC) and broad-spectrum antibacterials. In one study, CVCs were considered the main source of fungemia in more than 80% of cases (Lin *et al.*, 2005). A previous study conducted at the University Hospital of Maringá found a rate of yeast colonization of CVCs of 10.89% (Ramos *et al.*, 2011).

Treatment with two or more broad-spectrum antibacterials is associated to an imbalance in the endogenous flora which enables yeasts to overgrow (Lin *et al.*, 2005). Furthermore, some antibacterials are able to change the susceptibility profile of yeasts to antifungals by over expressing resistant genes (Vogel *et al.*, 2008; Oliver *et al.*, 2008) or by increasing their ability to form biofilms (McCool *et al.*, 2008).

Protein-synthesis targeted antibacterials, such as rifampicin and tetracycline, act on the antifungal activities of fluconazole. Rifampicin induces the activation of *Candida albicans* multi drug resistant-1 (MDR1) genes to fluconazole (Vogel *et al.*, 2008), while tetracycline alters the gene expression of ergosterol biosynthesis increasing resistance to fluconazole and terbinafine (Oliver *et al.*, 2008), while also increases *C. albicans* virulence, hyphae formation and, to a lesser extent, drug efflux and the formation of biofilms (McCool *et al.*, 2008).

Since the vast majority of ICU patients have CVCs inserted and receive at least one antibacterial for prophylaxis or treatment, the overgrowth of yeasts is a serious concern which may lead to a worsening of their diseases, prolonged hospitalization and increased mortality rates (Lin *et al.*, 2005).

The investigations of interactions of the antibacterials with the antifungals reported above only cite drugs that affect RNA and protein synthesis, rifampicin and tetracycline, respectively. However, no studies have been carried out with antibacterials that act on cell-wall synthesis.

Therefore, the objective of the present study was to evaluate, *in vitro*, whether yeast present in CVC colonizations, which have been exposed to antibacterials that act on bacterial cell-wall synthesis, undergo alterations in their susceptibility to fluconazole and voriconazole and in their ability to form biofilms.

MATERIAL AND METHODS

Material

The antibacterials cefepime (Aurobindo Pharma Limited), meropenem (Instituto BioChimico Indústria Farmacêutica Ltda), piperacillin-tazobactam and vancomycin (Novafarma Indústria Farmacêutica Ltda) were purchased by the University Hospital of Maringá, Brazil, and used in this research with permission. The antifungals fluconazole and voriconazole were purchased from Sigma Chemical Co.

The working concentrations of the antibacterials cefepime (24 mg/L), meropenem (12 mg/L), piperacillin-tazobactam (192 mg/L) and vancomycin (20 mg/L) were established according to their pharmacodynamic targets against: *Enterobacteria* ($3 \times \text{MIC} \leq 8 \mu\text{g/mL}$ for cefepime and $3 \times \text{MIC} \leq 4 \mu\text{g/mL}$ for meropenem), *Pseudomonas aeruginosa* ($4 \times \text{MIC} \leq 64/4 \mu\text{g/mL}$ for piperacillin-tazobactam) and *Staphylococcus aureus* ($5 \times \text{MIC} \leq 4 \mu\text{g/mL}$ for vancomycin), as established by the Clinical and Laboratory Standard Institute (CLSI, 2005), Mattoes *et al.* (2004) and Rybak (2006).

Sampling collection

The study protocol was approved by the Local Research Ethics Committee prior to sample collection (authorization number 251/07). Five yeasts isolated in a previous study (Ramos *et al.*, 2011) were included in this evaluation.

Antifungal susceptibility test

The minimal inhibitory concentrations (MIC) of fluconazole and voriconazole against the yeasts were obtained by broth microdilution according to the Clinical and Laboratory Standards Institute (CLSI, 2008). *C. parapsilosis* ATCC 22019 was used as a reference.

Antifungal susceptibility test of yeasts pre-treated with antibacterials

The yeasts were pre-treated with antibacterials by using a gradient of concentration system on slanted plates as described by Szybalski, Bryson (1952). Briefly, bases (10 mL) of Mueller-Hinton Agar (MHA) were solidified in the plates over slanted supports to provide inclined layers (30°) of culture medium, thus obtaining different thicknesses of medium. Second layers of MHA (10 mL) containing 10^6 colony-forming units per milliliter

(CFU/mL) of one of the yeast suspensions mixed with one of the antibacterials were added over the inclined layers and allowed to solidify to form flat surfaces. The plates were incubated at 35 °C for two days. The concentration of each antibacterial was therefore dependent on the thickness of the medium and the diffusion rate of the antibacterial.

The colonies which grew in the three regions of the antibacterial plates (high, intermediate and low concentration) were collected and tested for susceptibility to fluconazole and voriconazole, as described by CLSI, and for biofilm formation as described by Shin *et al.*, (2002).

A confirmatory test was carried out by mixing all the antibacterials at a fixed concentration and performing susceptibility tests against fluconazole and voriconazole for all of the yeasts.

Biofilm formation

To test for the formation of biofilms, suspensions of the yeast isolates, both treated and non-treated with antibacterials, were adjusted to a concentration of 3.7×10^7 CFU/mL and 20 μ L of each suspension was added to 96-well polystyrene microplates containing 180 μ L of Sabouraud Dextrose Broth (SDB) with glucose at a final concentration of 8%. The microplates were incubated at 35 °C for 24 h and the transmittance (%T) of each well was measured on an ASYS Expert Plus microplate reader at a wavelength of 405 nm. Wells containing only SDB without yeast suspensions were used as a negative control.

The degree of biofilm production was measured according to Shin *et al.*, 2002.

RESULTS AND DISCUSSION

C. tropicalis, *C. glabrata*, *C. albicans*, *C. parapsilosis* and *C. guilhermound*, previously isolated from CVC colonizations, were used to determine the influence of antibacterials on antifungal activity. All of the yeasts were susceptible to fluconazole and voriconazole, MIC ≤ 8 μ g/mL and MIC ≤ 1 μ g/mL, respectively, while the highest MIC value (8 μ g/mL) was for fluconazole against *C. glabrata*. For all of the yeasts tested, *in vitro* susceptibility to fluconazole and voriconazole after prior exposure to antibacterials did not differ significantly to that of the non-treated control groups (Table I). The additional susceptibility test, in which the antibacterials and antifungals were mixed together at the same time, produced the same results as those found in the antibacterial pre-treatment tests.

Many studies have emphasized that antibacterials induce a high risk of candidemia (Lin *et al.*, 2005). However, no studies have confirmed the lack of effect of antibacterials on antifungal drugs against *Candida* spp. The present research is the first study to have investigated possible interactions between cell-wall targeted antibacterials and fluconazole or voriconazole used against *Candida* spp.

Several studies have reported that prior treatment with antibacterials and fluconazole is a high-risk factor for candidemia and can induce resistant non-*albicans*

TABLE I – In vitro susceptibility test with voriconazole and fluconazole for *Candida* species pre-treated with cell-wall targeted antibacterials

Antifungals	Antibacterials	MIC (μ g/mL) values for <i>Candida</i>						
		<i>tropicalis</i>	<i>glabrata</i>	<i>guilliermondii</i>	<i>albicans</i>		<i>parapsilosis</i>	
					1	2	1	ATCC
Fluconazole	Non-treated Control	0.5	8	0.5	0.25	0.125	0.5	2
	V	0.25	8	1	0.25	0.125	0.5	2
	PT	0.5	8	0.5	0.25	0.125	0.5	2
	M	0.5	8	0.5	0.25	0.125	0.5	1
	C	0.25	8	0.5	0.25	0.125	0.5	1
Voriconazole	Non-treated Control	0.03	0.5	0.03	0.03	0.03	0.03	0.06
	V	0.03	0.5	0.03	0.03	0.03	0.03	0.03
	PT	0.03	0.25	0.03	0.03	0.03	0.03	0.03
	M	0.03	0.5	0.03	0.03	0.03	0.03	0.06
	C	0.03	0.5	0.03	0.03	0.03	0.03	0.03

Minimal Inhibitory Concentration (MIC); Vancomycin(V); Piperacillin-tazobactam (PT); Meropenem (M); Cefepime (C).

Candida (Lin *et al.*, 2005; Playford *et al.*, 2008). This increased resistance has been observed with candidemia caused by *C. glabrata* and *C. kruzei* after prolonged treatment with vancomycin and piperacillin-tazobactam (Lin *et al.*, 2005). The data from these studies suggests that antibacterials may, to some degree, affect antifungal activity and induce resistant genes in yeasts, as has been observed with rifampicin, doxycycline and tigecycline (Vogel *et al.*, 2008).

Rifampicin and tigecycline inhibit the synthesis of RNA and proteins, and induce the over expression of genes that are resistant to fluconazole (Vogel *et al.*, 2008; Oliver *et al.*, 2008). In the vast majority of studies, cell-wall targeted antibacterials are also included among those thought to produce a higher risk of candidemia as they are used extensively against nosocomial infections in clinical practice.

In fact, based on the mechanism of action of cell-wall targeted antibacterials, they are not expected to act on the cell walls of yeast since the compositions of the cell walls of bacteria and yeast are completely different. The critical target of cell-wall antibacterials is the peptidoglycan layer of bacteria, whereas the target of antifungals is the ergosterol layer of yeast.

The candidemia observed after prolonged antibacterial therapy seems to be more attributable to the patient’s general condition after long exposure to infection and a selection of resistant microbiota, or secondary to chronic renal disease and solid organ transplantation (Playford *et al.*, 2008).

Biofilm formation is an important virulence factor in fungal infections among ICU patients. Therefore, the influence of antibacterials on the ability of the yeast to form biofilms was also tested. As can be seen in Figure 1, *C. albicans* showed the greatest capacity to form biofilms whereas *C. tropicalis* showed the lowest, both for treated and non-treated samples. In this study, the ability to form biofilms was not influenced by the presence of the antibacterials, even at high concentrations. This finding is important, since biofilms are complex polymicrobial associations anchored to abiotic or biotic surfaces, embedded in extracellular matrix produced by the biofilms themselves, where they interact with each other and the environment. One of the main properties of biofilms is their capacity to be more resistant to antimicrobial agents than planktonic cells (Shin *et al.*, 2002).

These results demonstrate that cell-wall targeted antibacterials do not affect the biofilm-forming capacity

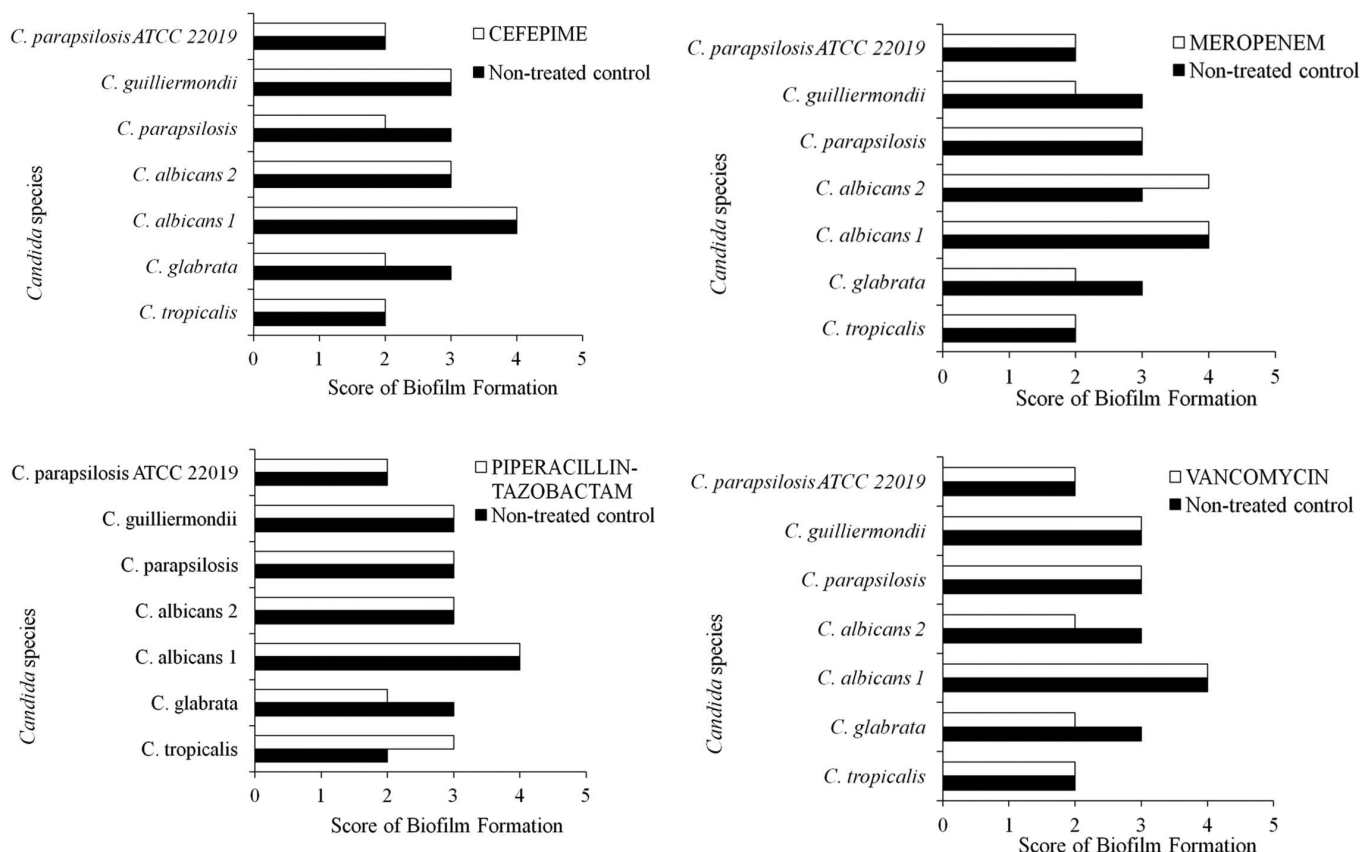


FIGURE 1 – Capacity of *Candida* species to form biofilm after treatment with antibacterials.

of *Candida* spp. Based on these results, it can be concluded that fungemia caused by *Candida* spp. is not directly influenced by the presence of cell-wall targeted antibacterials.

One limitation of this study is that the gene expression of *Candida* species treated with antibacterials was not explored. Nevertheless, this study reports a negative result which, from a clinical point of view, is important because it removes speculation about the possibility that cell-wall targeted antibacterials can induce candidemia by interfering with the antifungal activity of fluconazole and voriconazole.

Another speculative argument for antibacterials causing candidemia is that cell-wall targeted antibacterials may indirectly induce candidemia by potentiating host-directed inflammatory responses against invasive bacterial pathogens by releasing pro-inflammatory cell-wall components and intracellular toxins following bacteriolysis. This effect may exacerbate the inflammatory reactions damaging the host cell and tissues, worsening the patient's condition. However this same effect has not been observed with antibacterials such as macrolides, fluoroquinolones and tetracyclines, as these prevent the release of pro-inflammatory toxins of Gram positive and Gram-negative bacteria and also inhibit other virulence factors working against the excessive production of several potent inflammatory mediators (Anderson *et al.*, 2010). This difference in the mechanism of action of these classes of antibacterials may explain, in part, why all epidemiological risk factors point to the cell-wall targeted antibacterials and not to the protein-synthesis targeted antibacterials.

This study corroborates the results found by Playford *et al.*, (2008), who claimed that there was no correlation between antibacterial drugs exposure and infection by *Candida* spp. Using data from a large surveillance program, prior systemic antifungal exposure, prior gastrointestinal surgery, increasing age, and intravenous drug use were independently significant risk factors associated with non-candida and fluconazole-resistant *Candida* spp among ICU patients with candidemia, while pre-treatment with antibacterials were not significant.

CONCLUSIONS

In conclusion, this study proved that cell-wall targeted antibacterials do not affect *Candida* spp. growth and biofilm formation, and rejects the hypothesis that these antibacterials interact with antifungals to decrease their antifungal activity akin to protein-biosynthesis targeted antibacterials.

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