Cotrimoxazole enhances the in vitro susceptibility of *Coccidioides posadasii* to antifungals

Rossana de Aguiar Cordeiro[1,2]/+, Delia Jessica Astete-Medrano[2], Francisca Jakelyne de Farias Marques[1], Heuziwanne Tavares Leite Andrade[1], Lauro Vieira Perdigão Neto[1], Juliane Lira Tavares[3], Rita Amanda Chaves de Lima[1], Kharla Kharolyni Nobre Rabelo Patoilo[1], Andre Jalles Monteiro[4], Raimunda Sâmia Nogueira Brilhante[1,2], Marcos Fábio Gadelha Rocha[1,5], Zoilo Pires de Camargo[6], José Júlio Costa Sidrim[1,2]

[1]Centro Especializado em Micologia Médica [2]Programa de Pós-Graduação em Ciências Médicas [4]Departamento de Estatística, Universidade Federal do Ceará, Fortaleza, CE, Brasil [3]Departamento de Química [5]Programa de Pós-Graduação em Ciência Veterinária, Universidade Estadual do Ceará, Fortaleza, CE, Brasil [6]Departamento de Microbiologia, Imunologia e Parasitologia, Universidade Federal de São Paulo, São Paulo, SP, Brasil

The aim of the present study was to evaluate the effect of cotrimoxazole on the in vitro susceptibility of Coccidioides posadasii *strains to antifungals. A total of 18 strains of* C. posadasii *isolated in Brazil were evaluated in this study. The assays were performed in accordance with the Clinical and Laboratory Standards Institute guidelines and the combinations were tested using the checkerboard method. The minimum inhibitory concentrations were reduced by 11, 2.4, 4.3 and 3.5 times for amphotericin B, itraconazole, fluconazole and voriconazole, respectively. Moreover, it was seen that cotrimoxazole itself inhibited* C. posadasii *strains in vitro. The impairment of folic acid synthesis may be a potential antifungal target for* C. posadasii.

Key words: *Coccidioides posadasii* - cotrimoxazole - antifungals - susceptibility - antimicrobial synergism

In the recent years, several studies have shown the antifungal effect of "non-antifungal drugs" against true pathogenic species (Afeltra & Verweij 2003, Chapman et al. 2008, Cordeiro et al. 2009). The search for new antifungals is warranted because of the limited number of therapeutic drugs available for treating these infections. In addition, many researchers have been trying to improve the results obtained with antifungal monotherapy. Although data from controlled clinical trials are scarce, many recent reports have claimed beneficial effects of antifungal combinations for the treatment of severe fungal infections (Johnson & Perfect 2010, Matsuda et al. 2010).

Coccidioidomycosis is a deep fungal infection caused by the soil-dwelling ascomycetes *Coccidioides* spp. The disease has a broad clinical spectrum, ranging from a mild respiratory syndrome to progressive pneumonia and meningitis (Galgiani et al. 2005). Although *Coccidioides* spp rarely display antifungal resistance in vitro (Kriesel et al. 2008), specialists recognise that coccidioidomycosis is one of the most refractory fungal infections (Stevens et al. 2007). Therefore, several in vitro and in vivo studies have been performed in an attempt to identify new potential therapeutic drugs and antimicrobial combinations that can be used against *Coccidioides* spp (Shubitz et al. 2006, González et al. 2007, Cordeiro et al. 2009).

In this study, we investigated the effect of cotrimoxazole - a wide-spectrum antimicrobial formed by sulfamethoxazole (SMX) plus trimethoprim (TMP) - on the in vitro susceptibility of *Coccidioides posadasii* to amphotericin B (AMB), currently the most important drug used to treat life-threatening forms of coccidioidomycosis.

A total of 18 strains of *C. posadasii* isolated in the state of Ceará (Northeast Brazil) from clinical (n = 15) and environmental (n = 3) sources were evaluated in this study. The strains of *C. posadasii* were obtained from storage in 0.9% saline at 4ºC, subcultured on Sabouraud glucose agar (Difco, Detroit, USA) and incubated at 25ºC for 10 days. Prior to antimicrobial testing, the viability and purity of each isolate were evaluated by microscopic examinations and polymerase chain reaction experiments described elsewhere (Cordeiro et al. 2010). All procedures were performed within a class II biological safety cabinet in a biosafety level 3 laboratory.

Stock solutions of AMB (Sigma Chemical Co, USA), itraconazole (ITR) (Janssen Pharmaceutica, Belgium) and voriconazole (VRZ) (Pfizer Pharmaceuticals, USA) were prepared in dimethyl sulfoxide (Sigma Chemical Co, USA). Fluconazole (FLC) (Pfizer Pharmaceuticals, USA) was prepared in distilled water according to Clinical and Laboratory Standards Institute (CLSI 2008) SMX plus TMP (Hipolabor Ind Farmacêutica Ltda, Brazil) and SMX/TMP/AMB combinations were prepared in Roswell Park Memorial Institute (RPMI) 1640 me-

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+ Corresponding author: rossanacordeiro@ufc.br
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dium with L-glutamine and without sodium bicarbonate (Sigma Chemical Co, St. Louis, MO, USA) and buffered with 0.165 M MOPS (Sigma Chemical Co, USA). Serial two-fold dilutions of each antimicrobial combination were performed in RPMI 1640 medium.

Inoculum preparation was carried out as described by Cordeiro et al. (2009). Sterile normal saline was added to each agar slant and the cultures were gently scraped with cotton swabs. The suspension was transferred to a sterile tube and allowed to settle for 5 min and then the transmittance of the upper homogeneous supernatant was read at 530 nm and adjusted to 95% transmittance. The suspension containing arthroconidia and hyphae was diluted 1:10 with RPMI 1640 medium containing L-glutamine and without sodium bicarbonate and buffered to pH 7.0 with 0.165 M MOPS to obtain an inoculum of approximately 1 x 10^3-5 x 10^3 CFU/mL.

Antifungal susceptibility assays were performed by the broth macrodilution method (M38-A2) according to CLSI (2008) standards guidelines. First, the strains were tested against each antimicrobial alone to determine the minimum inhibitory concentrations (MICs). The drug concentration ranges tested were as follows: AMB, 0.0625-1.0 µg/mL; ITR, 0.0625-1.0 µg/mL; VRZ, 0.031-0.5 µg/mL; FLC, 0.78-12.5 µg/mL; SMX-TMP, 250/50-4,000/800 µg/mL. The procedures were repeated at least twice and each fungal strain was tested in duplicate. MICs for AMB and azoles were defined as the lowest concentration of the drug at which there was no visible fungal growth (CLSI 2008). We defined the MIC of SMX/TMP as the lowest drug concentration that caused 80% inhibition of visible fungal growth. After MIC definition for each drug, the strains were tested against the combination AMB/SMX/TMP. The following concentrations were tested: AMB, 0.007-0.116 µg/mL; SMX/TMP, 125/25-2,000/400 µg/mL. The MIC of each drug in combination was defined as the lowest concentration that caused 80% inhibition of visible fungal growth.

The non-parametric Wilcoxon Signed Rank Test was used for analysis of the antimicrobial combinations. The results were expressed as the mean and a p value of < 0.05 was considered significant.

TABLE I

Minimum inhibitory concentration (MIC) of sulfamethoxazole plus trimethoprim (SMX/TMP) and antifungals against clinical and environmental *Coccidioides posadasii* strains

	MIC (µg/mL)				
Strain	AMB	ITR	FLC	VRZ	SMX/TMP
Clinical source					
01-6-085	0.125	0.125	3.125	0.125	1,000/200
01-6-087	0.125	0.125	3.125	0.125	2,000/400
01-6-088	0.125	0.125	3.125	0.125	2,000/400
01-6-089	0.125	0.125	3.125	0.125	2,000/400
01-6-101	0.0625	0.125	6.25	0.125	2,000/400
01-6-102	0.125	0.125	6.25	0.125	2,000/400
01-6-103	0.125	0.5	6.25	0.125	1,000/200
05-02-063	0.125	0.125	6.25	0.25	2,000/400
05-02-064	0.125	0.25	6.25	0.25	2,000/400
05-02-065	0.125	0.125	6.25	0.125	2,000/400
05-02-066	0.0625	0.25	6.25	0.25	2,000/400
05-02-067	0.125	0.125	3.125	0.125	2,000/400
05-02-068	0.125	0.125	6.25	0.125	1,000/200
05-02-069	0.125	0.125	6.25	0.125	1,000/200
05-02-070	0.0625	0.125	6.25	0.125	2,000/400
Environmental source					
01-6-090	0.125	0.25	6.25	0.125	2,000/400
01-6-091	0.125	0.125	3.125	0.125	2,000/400
01-6-092	0.125	0.125	6.25	0.125	2,000/400
Geometric mean	0.111	0.151	4.961	0.140	1714.48/342.89

AMB: amphotericin B; FLC: fluconazole; ITR: itraconazole; VRZ: voriconazole.

The MIC (geometric means) was 0.111 µg/mL, 0.155 µg/mL, 4.819 µg/mL and 0.142 µg/mL for AMB, ITR, FLC and VRZ, respectively. The antimicrobial combination SMX/TMP was also able to inhibit the growth of all *C. posadasii* strains in vitro and the MIC values ranged from 1,000/200-2,000/400 µg/mL (Table I). When used in combination with SMX/TMP, the MICs of the antifungals were reduced by 11, 2.4, 4.3 and 3.5 times for AMB, ITR, FLC and VRZ, respectively ($p < 0.05$) (Table II). Sulpha derivatives are antimetabolic drugs that impair folic acid synthesis in microorganisms. These compounds have a broad antimicrobial spectrum and are active against several aerobic bacteria and some protozoan species and these compounds are frequently used successfully for the treatment and prevention of pneumonia caused by *Pneumocystis jiroveci* in acquired immune deficiency syndrome patients (Thomas et al. 2009). Although these compounds are not common first-line drugs in the management of fungal infections, the combination of SMX/TMP has shown good results in treating paracoccidioidomycosis (Hahn et al. 2003, Wanke & Aidê 2009). Our results show that SMX/TMP was able to enhance the susceptibility of *C. posadasii* to AMB by reducing the AMB MIC by approximately 5.85 times. Surprisingly, it was shown that the combination SMX/TMP alone was also able to impair *C. posadasii* growth in vitro.

In a previous work, Hanafy et al. (2007) found a great variability among SMX MICs against *Cryptococcus* sp. The authors reported that higher MIC values were attained when susceptibility tests were performed in organic-rich media because para-aminobenzoic acid (PABA) or PABA-related compounds may be present. In this study, we tried to perform the susceptibility tests with yeast nitrogen base, as suggested by Hanafy et al. (2007), but unfortunately this medium did not support the growth of *C. posadasii* (data not shown). Because other studies have already been performed with RPMI (Yekutiel et al. 2004, Navarro-Martinez et al. 2006), we decided to evaluate this medium in our experiments. However, we assumed that in PABA-free medium, SMX/TMP may cause a greater reduction in AMB MICs.

We hypothesise that low concentrations of AMB may have caused slight damage to the fungal plasmatic membrane, allowing moderate ion leakage. In addition, these channels across the membrane may have allowed SMX/TMP to enter the cell. Therefore, we believe that acid folic blockade may be a potential target for *C. posadasii* inhibition. The design of sulpha drugs with higher affinity for dihydrofolate reductase or other folic acid biosynthetic pathway enzymes could enhance fungal in-

TABLE II

Minimum inhibitory concentration (MIC) of sulfamethoxazole plus trimethoprim (SMX/TMP) associated with antifungals against clinical and environmental *Coccidioides posadasii* strains

	MIC (µg/mL)							
Strains	SMX/TMP + AMB		SMX/TMP + ITR		SMX/TMP + FLC		SMX/TMP + VRZ	
01-6-085	250/50	0.007	250/50	0.062	250/50	0.781	500/100	0.062
01-6-087	500/100	0.015	500/100	0.125	250/50	0.781	250/50	0.031
01-6-088	500/100	0.015	500/100	0.125	500/100	1.562	250/50	0.031
01-6-089	500/100	0.015	250/50	0.062	250/50	0.781	250/50	0.031
01-6-090	500/100	0.015	500/100	0.125	500/100	1.562	500/100	0.062
01-6-091	500/100	0.015	250/50	0.062	500/100	1.562	500/100	0.062
01-6-092	500/100	0.015	250/50	0.062	250/50	0.781	250/50	0.031
01-6-101	500/100	0.015	500/100	0.125	500/100	1.562	500/100	0.062
01-6-102	1,000/200	0.031	250/50	0.062	500/100	1.562	500/100	0.062
01-6-103	500/100	0.015	250/50	0.062	500/100	1.562	250/50	0.031
05-02-063	500/100	0.015	250/50	0.062	250/50	0.781	250/50	0.031
05-02-064	500/100	0.015	250/50	0.062	250/50	0.781	250/50	0.031
05-02-065	1,000/200	0.031	500/100	0.125	250/50	0.781	500/100	0.062
05-02-066	500/100	0.015	250/50	0.062	500/100	1.5625	250/50	0.031
05-02-067	500/100	0.015	500/100	0.125	500/100	1.562	500/100	0.062
05-02-068	500/100	0.015	250/50	0.062	250/50	0.781	250/50	0.031
05-02-069	500/100	0.015	250/50	0.062	250/50	0.781	250/50	0.031
05-02-070	1,000/200	0.031	250/50	0.062	500/100	1.562	500/100	0.062

AMB: amphotericin B; FLC: fluconazole; ITR: itraconazole; VRZ: voriconazole.

hibition. In fact, this pathway seems to be determinant of fungal viability, as *Aspergillus fumigatus* mutants defective in folate biosynthesis have been shown to be avirulent (Brown et al. 2000).

According to Navarro-Martinez et al. (2006), interruption of the folic acid biosynthetic pathway also impairs ergosterol production in *Candida albicans*, possibly by disturbing the metabolism of sterol C24 methyltransferase because the synthesis of its substrate is also blocked. It is well-known that ergosterol biosynthesis is also interrupted by azole antifungals agents that act on sterol 14 α-demethylase enzyme. The synergistic effect of these drugs was demonstrated in this study.

Although it is reasonable to suppose that the results of susceptibility tests against the parasitic form of *C. posadasii* are more reliable, very few studies have tested the susceptibility pattern of the yeast-like form of the fungus because the transition from mycelia to yeast it is not easily achieved (Hector et al. 1988). In contrast, tests with cells in the saprophytic phase are easier to perform and, even though CLSI protocols are not intended for testing dimorphic fungi, these protocols can be applied, generating results that are easy to compare.

The results obtained in this study demonstrate the potential of the SMX/TMP/AMB combination, which has an inhibitory effect on *C. posadasii* strains, even at sub-MIC concentrations of AMB. In addition, the results showed that SMX/TMP also has an inhibitory effect on *C. posadasii*, suggesting that acid folic blockade may be a potential antifungal target for *C. posadasii*. As far as we know, this is the first report of the antifungal potential of sulpha drugs against this pathogen.

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