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Screening of antifungal susceptibility in cave-dwelling aspergilli and report of an amphotericin B-resistant *Aspergillus flavus*

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Abstract: Caves are stable environments that favour the development of several microorganisms. The aspergilli represent a large number of species isolated from caves including strains capable of causing serious invasive opportunistic infections in humans. Considering that caves may harbour resistant strains to many antibiotics, investigation on the response of opportunistic aspergilli, isolated from pristine and tourist caves to antifungal agents and the mechanisms involved in resistance might be clinically relevant. A total of 32 strains of the species *Aspergillus candidus*, *A. flavus*, *A. fumigatus*, *A. niger*, *A. tamarii*, and *A. terreus* were isolated from caves in the iron quadrangle in Brazil. The strains were tested for their susceptibility to amphotericin B (AMB), itraconazole, voriconazole and terbinafine. One strain was analysed for the mechanism involved in the AMB-resistance, i.e., ergosterol content, lipid peroxidation and enzymatic activity of the antioxidant system. Terbinafine minimum inhibitory concentrations (MICs) ranged between 0.003 and 1.0 µg/mL; voriconazole MICs ranged between 2.0 and >16.0 µg/mL; itraconazole MICs ranged between 0.25 and 8.0 µg/mL and amphotericin B MICs ranged between 0.03 and 4.0 µg/mL. The AMB-resistant strain of *A. flavus* was detected with MIC value of 4 µg/mL. Resistance to AMB relied on higher ergosterol levels and increased enzymatic activity of the peroxidase and superoxide-dismutase, with lower lipid peroxidation. These results enhance the knowledge of natural antifungal resistance in the subterranean ecosystem, and broaden the knowledge about the subterranean microbiota.

Keywords: Iron cave, *Aspergillus flavus*, antifungal drugs, amphotericin B, primary resistance

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INTRODUCTION

Caves are unique ecosystems with environmental characteristics that may favour the growth of several microorganisms (Poulson & White, 1969; Culver & White, 2005). The genus *Aspergillus* has often been reported in subterranean environments and represents many of the identified fungal species isolated from caves (Vanderwolf et al., 2013), including several species capable of causing serious invasive opportunistic infections in humans (Taylor et al., 2013; Vanderwolf et al., 2013; Taylor et al., 2014). The species *Aspergillus fumigatus* (Perfect et al., 2001), *A. flavus* (Walsh et al., 2008), *A. terreus* (Iwen et al., 1998; Mokaddas et al., 2010), and *A. niger* (Person et al., 2010) are the main causative agents of invasive pulmonary aspergillosis (IPA) in humans (Walsh et al., 2008; Kousha et al., 2011). Although IPA occurs mainly among immunocompromised individuals, cases of pulmonary infection by aspergilli have been reported in immunocompetent individuals (Person et al., 2010; Kousha et al., 2011). The polyenes and azoles are the agents most commonly used in the treatment of IPA. Resistance to these drugs have been increasingly reported (Ghannoum & Rice, 1999; Singh, 2001; Kousha et al., 2011) raising the possibility of natural acquired resistance (Loeffler & Stevens, 2003).

Most caves are considered extreme environments due to their oligotrophic conditions. Normally, cave-

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dwelling organisms adapt to this nutrient limitation in order to survive and colonize the ecosystem (Culver & White, 2005). Among cave microorganisms, this adaptation may occur at the morphological and/or physiological level. Competition for substrate among sympatric fungal species normally leads to production of metabolites capable of eliminating competition, such as antibiotics, acidifying metabolites, and toxins (Griffin, 1994). Antibiotic resistance among cave isolated bacteria has been described as result of genetic background and selective forces involved in various ecological interactions in the subterranean environment (Bhullar et al., 2012). Fungi may also be undergoing the same process, which may cause natural selection of opportunistic and pathogenic strains resistant to currently used antifungal drugs. The fact that several caves have large mats of actinomycetes competing with fungi for substrates and that the most potent antifungal drug (amphotericin B) used in treatment of IPA was firstly obtained from actinomycetes (*Streptomyces nodosus*) (Svahn et al., 2015), reinforces this hypothesis. Thus, prospective studies on the effect of antifungal drugs against strains colonizing caves are relevant.

Considering that IPA may represent a silent emerging risk to cave visitors, and that caves may harbour strains resistant to antifungals, we conducted a pioneer investigation on the response of opportunistic *Aspergillus* spp., isolated from pristine and tourist caves, to the most commonly used antifungal agents (amphotericin B, itraconazole, voriconazole and terbinafine) and the mechanisms involved in the resistance to AMB.

MATERIALS AND METHODS

Study site

The strains used in this study were obtained from eight different caves located in the Iron Quadrangle (Quadrilátero Ferrífero, Minas Gerais state, Brazil). This region is economically important due to their proximity with the state capital, urban/rural areas and sites with large mining activities (mainly metal ores). Furthermore, cave visitation has increased in past years. The strains used in this study were isolated from seven iron ore caves: Rola Moça II (Brumadinho, Minas Gerais, Brazil- 20°03'38.5" S / 44°00'30.4" W), Rola Moça III (Belo Horizonte, Minas Gerais, Brazil- 20°02'38.1" S / 44°00'23.2" W), Macumba (Sabará, Minas Gerais, Brazil- 19°40'01.1" S / 43°40'30.1" W), Romeiros (Sabará, Minas Gerais, Brazil- 19°49'00.3" S / 43°40'35.9" W), Bloco Abatido (Sabará, Minas Gerais, Brazil- 19°49'02" S / 43°40'50" W), Cascalhinho (Caeté, Minas Gerais, Brazil- 19°48'59.1" S / 43°40'28.7" W), Eremita (Sabará, Minas Gerais, Brazil- 19°49'19.5" S / 43°40'49.5" W) and one dolomitic cave, Nossa Senhora da Conceição da Lapa (Ouro Preto, Minas Gerais, Brazil- 20°18'18.4" S / 43°28'20.6" W) (Fig. 1).

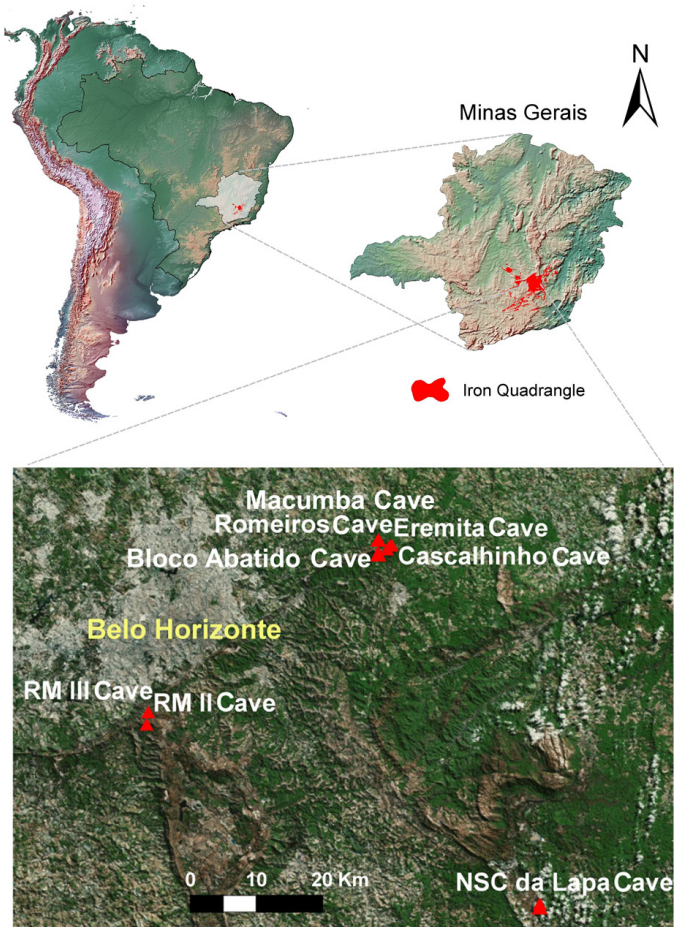


Fig. 1. Location of the eight caves studied in the Iron Quadrangle (Minas Gerais State, Brazil): Rola Moça II Cave, Rola Moça III Cave, Macumba Cave, Romeiros Cave, Cascalhinho Cave, Eremita Cave, Bloco Abatido Cave, and Nossa Senhora da Conceição da Lapa Cave. State capital Belo Horizonte is also indicated.

Aspergillus strains

Five species of aspergilli were selected due to their association as causative agents of IPA in humans. A total of 33 strains of the species *Aspergillus candidus* (Taylor et al., 2014), *A. flavus* (Ghannoum & Rice, 1999), *A. fumigatus* (Perfect et al., 2001), *A. niger* (Perfect et al., 2001), *A. terreus* (Culver & White, 2005), and *A. tamarii* (Culver & White, 2005) were used that had previously been isolated from the caves during a mycological inventory performed in the period between the years 2010/2013. Strains were sampled from air by the settle plate technique with a 20-minute exposure on Dichloram Rose Bengal Cloramphenicol Agar (DRBC, Acumedia Laboratories, Lansing, MI USA) and Sabouraud Dextrose Agar (SDA, Difco Laboratories, Detroit, MI USA) with cloramphenicol (0.1%), and from sediment by a 10-fold serial dilution on DRBC (Acumedia). These strains are currently deposited in the mycological collection of cave fungi in the Mycological Laboratory of the Federal University of Minas Gerais (Laboratório de Micologia da Universidade Federal de Minas Gerais-LM/UFMG, Belo Horizonte, Minas Gerais, Brazil).

Identification of species was confirmed through analysis of macro and micromorphological characteristics in solid media, following standard taxonomic keys and recently published works (Klich, 2002; Domsch et al., 2007). Two media were used for each strain and at different temperature regimens: Czapeck Yeast Extract Agar (CYA, Labsynth, Diadema, SP Brazil) at 25°C/7d; CYA (Labsynth) at 37°C/7d; and Malt Extract Agar (MEA, Difco) at 25°C/7d (Klich, 2002).

Thermotolerance assay

Before initiating the *in vitro* susceptibility assays, a preliminary triage was performed to exclude strains with negative growth at 37°C. The media used in this procedure were SDA (Difco) (25°C and 37°C for 7days) and Brain Heart Infusion Agar (BHIA, Acumedia) (37°C for 7 days). The strains presenting positive growth at 37°C were submitted to *in vitro* drug susceptibility assays to amphotericin B (AMB), itraconazole (ITC), voriconazole (VCZ) and terbinafine (TBF).

Minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentration (MIC) for AMB (Sigma-Aldrich, St. Louis, Missouri, USA), ITC (Sigma-Aldrich), VCZ (Sigma-Aldrich) and TBF (Sigma-Aldrich) were determined by the antifungal microdilution susceptibility standard test proposed by the CLSI M38-A2 (CLSI, 2008). The inoculum was prepared in sterile saline solution and the transmittance of suspension was adjusted to 68-70% (530 nm) as standard protocol for filamentous fungi (M38 from CLSI). This suspension was diluted in RPMI-1640 buffered with MOPS (Sigma-Aldrich) medium to achieve 1.0 x 10^3 to 5.0 x 10^3 CFU/mL (colony forming units per mL). The drugs were dissolved in 100% dimethylsulphoxide according to the protocol of CLSI at 1.0 mg/mL. Final concentrations ranged from 0.03 to 16 µg/mL for AMB, ITC and VCZ and ranged from 0.00195 to 1.0 µg/mL for TBF.

In each case, a volume of 100 µL of the inoculum suspension was transferred to sterile flat-bottom 96 well plates containing100 µL of each of the antifungal. Control on RPMI-1640 was also performed (control growth). The plates were incubated at 37°C for 72h. All tests were performed in duplicate (for each strain).

Ergosterol quantification

Amphotericin B resistant (AmB-R) and amphotericin B susceptible (AmB-S) strains were cultured (37°C/72h) on SDA supplemented with AmB (1/2 MIC concentration) and approximately 10.0 mg of the fungal cell mass were transferred to polypropylene tubes. A growth control was also performed. For the extraction of lipids, 3 mL of ethanolic solution of potassium hydroxide 25% were added to each cell mass and agitated for one minute. The tubes were incubated in a water bath at 85°C for one hour and cooled at room temperature. A mixture of 1 mL of sterile water and 3 mL of n-heptane (Sigma-Aldrich) was added, followed by agitation in a vortex for three minutes. The supernatant was removed and the reading was performed in spectrophotometer at 282 nm and 230 nm. A calibration curve with standard ergosterol (Sigma-Aldrich) was constructed and used to calculate the amount of ergosterol. The results were expressed as percentage of ergosterol in comparison with the growth control and represent the means of three independent experiments.

Lipid peroxidation assay

Aspergillus strains were cultured (37°C/72h) on SDA supplemented with amphotericin B (1/2 MIC concentration) and hydrogen peroxide (HP) 1% as positive control. The products of the lipid peroxidation were measured as thiobarbituric acid-reactive substances (TBARS). The pellet was frozen and homogenized with 1000 µL in ice-cold 1.1% phosphoric acid using water as diluent (v/v). Four hundred µL of the homogenate was mixed with 400 µL of 1% thiobarbituric acid (Sigma-Aldrich) prepared in 50 mM NaOH containing 0.1 mM butylated hydroxytoluene and 200 µL of 7% phosphoric acid (all the solutions were kept on ice during manipulation). Subsequently, samples (pH 1.5) were heated for 60 minutes at 98°C and 1500 µL of butanol were added. The mixture was mixed vigorously using a vortex and centrifuged for five minutes at 2000 g. The organic layer was transferred and the absorbance was measured at 532 nm (Termo Scientific Multiscan spectrum, Termo Ficher Scientific). The thiobarbituric acid solution was replaced by 3 mM HCl in the controls. TBARS values were calculated using the extinction coefficient of 156 mM/cm and represent the means of three independent experiments. The results were expressed as the ratio between TBARS from cells treated with AMB or HP and the values registered in the control ± SE.

Enzymatic activity of the antioxidant system

Prior to the tests, a cell-free extract from susceptible and resistant strains of *A. flavus* were cultured (37°C/72 h) on SDA supplemented with AMB (1/2 MIC concentration) and on positive control with HP 1% according to the method described by Ferreira et al. (2013). Soluble protein was determined using the Bradford test using a standard curve of bovine serum albumin.

Peroxidase (PER) activity

The PER activity was analyzed according with method previously used (Ferreira et al., 2013) and measured in a system containing H_2O_2 and guaiacol as substrate. Values were obtained by monitoring the absorbance changes at λ = 470 nm using molar extinction coefficient value of 26.61 $M^{-1}cm^{-1}$ for the product tetra guaiacol formed by the enzymatic reaction. The data were calculated in nmols/min mg^{-1} of protein and represent the means of three independent experiments. The results were expressed as the ratio of nmols/min mg^{-1} of protein from cells treated with AMB or HP to the nmols/min mg^{-1} of protein from growth control ± SE.

Superoxide dismutase (SOD) activity

The SOD activity was measured by the inhibition of pyrogallol autoxidation as described previously (Ferreira et al., 2013). In the test samples, 100 µL of cell-free extract was added to pyrogallol and the inhibition of autoxidation was monitored every 30s for three minutes at a wavelength of 420 nm. The unit of SOD was considered as pyrogallol autoxidation per 200 µL calculated as follows:

Unit of SOD mL^{-1} of sample = (A-B)/A x 50 x 100 x 0.6 (dilution factor) where A is the difference in absorbance per minute in the control and B the difference in

absorbance per minute in the test samples. Data was calculated in units/mg of protein and represent the means of three independent experiments. The results were expressed as the ratio between the units/mg of protein extracted from cells treated with AMB or HP and the units/mg of protein from growth control ± SE.

Statistical analysis

A Student's two-tailed *t* test and a one-way analysis of variance (ANOVA) with the Bonferroni multiple-comparison were used to compare the differences among groups. Significance was determined by values of $p<0.05$ in all experiments. The program used was GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA, USA).

RESULTS

A total of 33 strains of *Aspergillus* spp. were isolated in this study (Table 1). Thirty-two were able to grow at 37°C. The results of growth and brief characterization of their habitat are shown in Table 1.

In vitro susceptibility data

The MICs for azoles against the *Aspergillus* strains ranging between 2 µg/mL and >16 µg/mL to VCZ, and

Table 1. *Aspergillus* strains isolated from caves in the Iron Quadrangle in Minas Gerais state, Brazil Growth rate of *Aspergillus* spp. on Sabouraud Dextrose Agar (25°C and 37°C) and Brain Heart Infusion (37°C) after seven days of incubation.

Taxon (strain no.)	Growth (mm)			Habitat		
	SDA		BHI	Substrate	Zone of isolation	Cave
	25°C	37°C	37°C			
Aspergillus candidus						
UFMGLMCC03	17	8	3	Air	Aphotic	RM 3
UFMGLMCC028	20	12	11	Sediment	Disphotic	RM 3
UFMGLMCC011	20	5	3	Sediment	Disphotic	Gruta do Bloco Abatido
UFMGLMCC015	19	18	15	Sediment	Aphotic	Gruta do Cascalhinho
UFMGLMCC014	22	-	-	Air	Disphotic	RM3
Aspergillus flavus						
UFMGLMCC04	60	51	50	Air	Disphotic	Gruta do Eremita
UFMGLMCC05	62	50	50	Sediment	Disphotic	RM3
UFMGLMCC06	53	47	45	Sediment	Disphotic	Gruta da Macumba
UFMGLMCC09	62	44	49	Air	Photic	Gruta dos Romeiros
UFMGLMCC019	61	60	60	Air	Disphotic	Gruta N. S. Conceição
UFMGLMCC021	59	55	52	Sediment	Aphotic	Gruta N. S. Conceição
UFMGLMCC024	52	49	44	Sediment	Disphotic	Gruta do Cascalhinho
UFMGLMCC025	63	62	63	Sediment	Disphotic	Gruta RM2
UFMGLMCC026	70	66	65	Sediment	Photic	Gruta Eremita
UFMGLMCC027	58	55	59	Sediment	Disphotic	Gruta RM3
UFMGLMCC030	69	58	59	Sediment	Aphotic	Gruta N. S. Conceição
UFMGLMCC033	44	49	50	Vegetal debris	Disphotic	Gruta dos Romeiros
Aspergillus fumigatus						
UFMGLMCC01	61	59	62	Air	Aphotic	RM 3
UFMGLMCC02	64	60	58	Sediment	Disphotic	Gruta N. S. Conceição
UFMGLMCC07	57	56	59	Sediment	Aphotic	Gruta do Bloco Abatido
UFMGLMCC013	68	62	59	Sediment	Disphotic	Gruta RM3
UFMGLMCC010	66	69	67	Sediment	Disphotic	Gruta do Cascalhinho
UFMGLMCC022	64	60	61	Sediment	Disphotic	Gruta N. S. Conceição
Aspergillus niger						
UFMGLMCC016	70	68	68	Sediment	Disphotic	RM3
UFMGLMCC017	54	52	53	Sediment	Disphotic	Gruta RM2
UFMGLMCC018	71	64	61	Air	Disphotic	Gruta do Eremita
UFMGLMCC023	69	68	68	Air	Disphotic	Gruta do Eremita
UFMGLMCC029	57	49	52	Sediment	Aphotic	Gruta N. S. Conceição
UFMGLMCC034	62	60	62	Sediment	Disphotic	Gruta da Macumba
Aspergillus tamarii						
UFMGLMCC012	49	36	39	Sediment	Aphotic	Gruta N. S. Conceição
UFMGLMCC020	53	53	51	Sediment	Disphotic	Gruta dos Romeiros
Aspergillus terreus						
UFMGLMCC031	59	54	50	Sediment	Disphotic	Gruta do Bloco Abatido
UFMGLMCC032	62	59	55	Sediment	Disphotic	Gruta RM2

between 0.25 and 8 µg/mL to ITC. Itraconazole had lower MIC values, with at least 50% of the *Aspergillus* strains inhibited at 0.5 µg/mL. The lower ITC MICs were observed against *A. candidus* and *A. terreus* (Table 2).

Amphotericin B had low MICs against the *Aspergillus* species tested, being the values below 2 µg/mL for 29 strains. However the AMB MIC for three strains were 2 µg/mL (*A. flavus, A. niger,* and *A. terreus*) e.g., breakpoint determined by the CLSI; and 4 µg/mL for one strain of *A. flavus*. The mean value for AMB MIC50 was 0.5 µg/mL against *A. candidus*, *A. flavus*, *A. fumigatus*, *A. tamarii*, and *A. terreus*. For *A. niger* this value was lower with MIC of 0.25 µg/mL for at least fifty per cent of strains. The strain UFMGLMCC05 (*A. flavus*) was classified as amphotericin B-resistant (AmB-R) and submitted to further analysis of resistance mechanism. Finally, TBF MIC values ranged between 0.003 and 1 µg/mL (Table 2).

Table 2. Minimal Inhibitory Concentration (MIC) values (µg/mL) for amphotericin B (AMB), itraconazole (ITC), voriconazole (VCZ) and terbinafine (TBF) against thirty-two *Aspergillus* strains isolated from caves in Minas Gerais (Brazil).

Taxon (strain no.)	MIC (µg/mL)			
	AMB	**ITC**	**VCZ**	**TBF**
Aspergillus candidus				
UFMGLMCC03	1	0.5	8	0.0625
UFMGLMCC028	0.03	0.25	4	0.0625
UFMGLMCC11	0.5	0.5	4	0.125
UFMGLMCC15	0.5	0.5	4	0.003
MIC 50 (µg/mL)[a]	**0.5**	**0.5**	**4**	**0.0625**
Aspergillus flavus				
UFMGLMCC04	0.5	4	4	0.125
UFMGLMCC05	4[b]	0.25	8	0.0625
UFMGLMCC06	0.5	4	>16[b]	0.0625
UFMGLMCC09	1	0.5	8	0.125
UFMGLMCC019	0.5	4	4	0.0312
UFMGLMCC021	0.5	0.5	8	0.0625
UFMGLMCC024	0.5	1	>16[b]	0.5
UFMGLMCC025	1	0.5	8	1[b]
UFMGLMCC026	1	0.5	8	0.0625
UFMGLMCC027	0.25	1	4	0.0625
UFMGLMCC030	2	4	4	0.001
UFMGLMCC033	1	8	16[b]	0.5
MIC 50 (µg/mL)	**0.5**	**1**	**8**	**0.0625**
Aspergillus fumigatus				
UFMGLMCC01	1	4	8	1
UFMGLMCC02	0.5	1	2	0.0625
UFMGLMCC07	1	4	4	0.125
UFMGLMCC013	0.5	0.5	4	0.0625
UFMGLMCC010	0.25	0.5	4	0.0625
UFMGLMCC022	0.5	4	8	0.003
MIC 50 (µg/mL)	**0.5**	**1**	**4**	**0.0625**
Aspergillus niger				
UFMGLMCC016	0.25	8	4	0.0625
UFMGLMCC017	0.25	8	4	0.001
UFMGLMCC018	0.25	8	8	0.0625
UFMGLMCC023	0.5	0.5	8	0.003
UFMGLMCC029	0.0125	0.25	8	0.0625
UFMGLMCC034	2	4	8	0.0625
MIC 50 (µg/mL)	**0.25**	**4**	**8**	**0.0625**
Aspergillus tamarii				
UFMGLMCC012	0.5	1	4	0.03
UFMGLMCC020	1	1	8	0.03
MIC 50 (µg/mL)	**0.5**	**1**	**4**	**0.03**
Aspergillus terreus				
UFMGLMCC031	2	0.5	4	0.25
UFMGLMCC032	1	0.25	4	0.125
MIC 50 (µg/mL)	**1**	**0.25**	**4**	**0.125**

[a]MIC50: MIC value which inhibits 50% of the strains

Amphotericin B-resistant versus susceptible *A. flavus*

AmB-R *A. flavus* (UFMGLMCC05) was collected from the sediment in the disphotic zone of RM3 cave where whitish/yellowish mats of actinomycetes (including *Streptomyces* spp., unpublished data) covered the sediment. The AmB-S used for comparison was obtained from our mycological collection and was firstly isolated from a highly visited show cave with no visible growth of actinomycetes (*Aspergillus flavus* UFMGLMCC103, Maquiné Cave/Cordisburgo, MG, Brazil). We have selected this AmB-S strain to eliminate possible local adaptive effects.

The AmB-R has higher levels of ergosterol than and AmB-S *A. flavus* in control media ($p<0.05$) and when exposed to AMB ($p<0.05$) (Fig. 2). Furthermore, the lipid peroxidation results (expresses as TBARS levels) showed that the AmB-R strain was less susceptible to oxidative stress caused by AMB and HP ($p<0.05$) (Fig. 3). The investigation of enzymatic activities of PER and SOD in response to oxidative stress showed that PER activity was similar between the AmB-S and AmB-R when treated with AMB. When treated with HP, AmB-S had lower PER activity compared with a large increase in AmB-R strain (Fig. 3) ($p<0.05$). On the other hand, the SOD activity was higher ($p<0.05$) in the AmB-R strain exposed to the drug and HP (Fig. 3).

DISCUSSION

Resistance to antibiotic compounds involve several mechanisms aimed at reducing drug content in the cell, decreasing interaction between the drug and its target, and metabolic modifications to counter balance the lethal effect of the drug (Vandeputte et al., 2012). It is evident that all mechanisms involved in drug-resistance result from an adaptive response and fitness enhancement (Anderson, 2005). This interaction is commonly triggered by contact with the drug due to soil/water contamination (clinical or agricultural use) or ecological interactions with organisms producing these antifungal agents in the environment (Anderson, 2005; Vandeputte et al., 2012). Although recent studies have focused in investigating drug resistance and mechanisms involved among cave isolated bacteria (Bhullar et al., 2012), this is the first study that aimed at investigating drug resistance among cave isolated filamentous fungi.

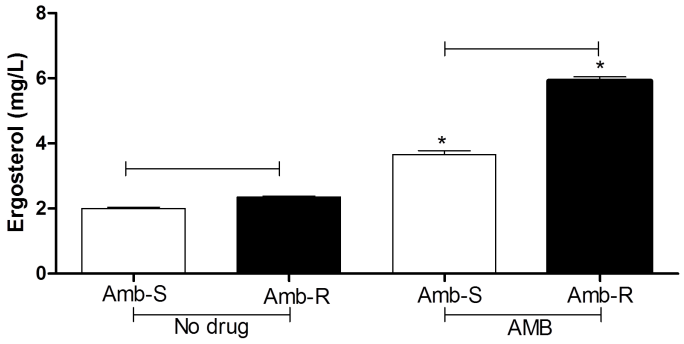
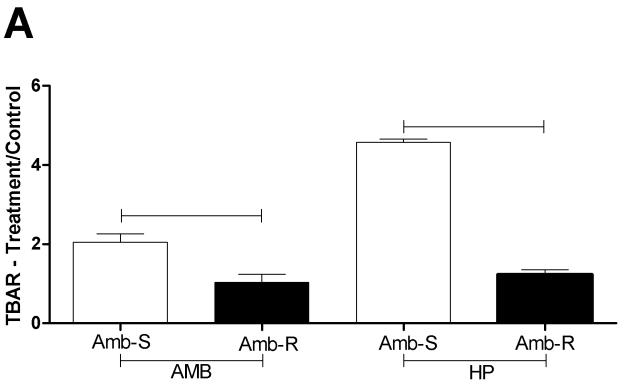


Fig. 2. Effect of amphotericin B (AMB) in susceptible and resistant *Aspergillus flavus* strains. Results are expressed in mg/mL. Data are represented as the mean + SEM of two independent experiments in triplicate assays. Statistically significant differences between the treatment and the control (no drug) are indicated with connect lines and between susceptible and resistant strains are indicated with asterisks ($p<0.05$). Amb-S = susceptible strain to amphotericin B; Amb-R = resistant strain to amphotericin B.



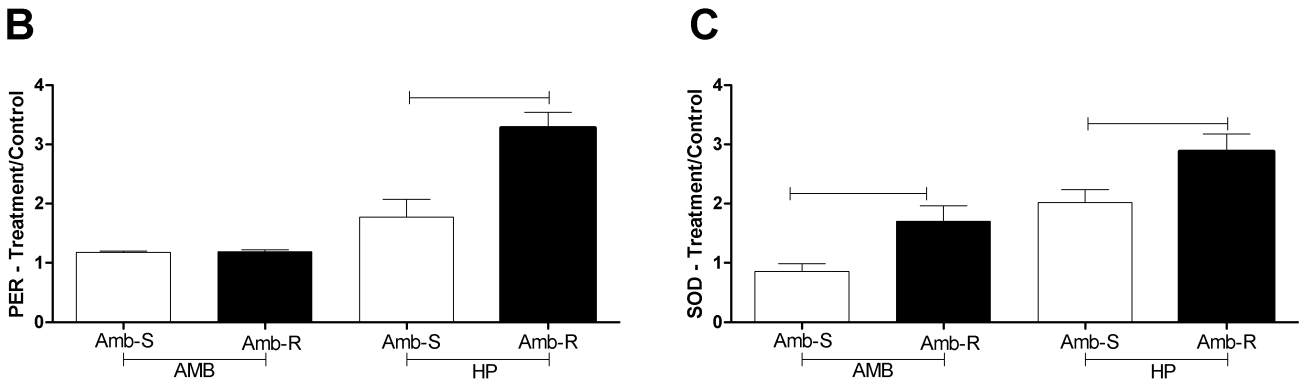


Fig. 3. Resistant cells are less susceptible to oxidative burst. (A) Lipid peroxidation (TBARs, thiobarbituric acid-reactive substances) (B) PER (peroxidase) (C) SOD (superoxide dismutase) activities after treatment with amphotericin B (AMB) and hydrogen peroxide (HP). Results are expressed as the ratio of the treatment to the control. Statistical differences are represented by the connect line ($p < 0.05$). Data represent the mean ± S.E. of two independent experiments consisting of triplicate assays. Amb-S = susceptible strain to amphotericin B; Amb-R = resistant strain to amphotericin B.

Terbinafine had the lowest MICs among the cave isolated fungi and the lower MICs with no resistant strains. Although TBF is not usually used in treatment of invasive pulmonary aspergillosis (Kousha et al., 1999), its use combined with AMB and azoles has been investigated (Ryder & Leitner, 2001; Mosquera et al., 2002; Cuenca-Estrella, 2004; Fernandez et al., 2015). Additive and synergistic relations have been reported between triazoles and TBF, as well as sinergism between AMB and TBF (Singh & Paterson, 2005). Additionally the AmB-resistant *A. flavus* had low TBF MICs, which is a result that deserves further evaluation aimed at combined use of drugs.

Our results clearly showed that, among the azoles, ITC had lower MIC values against the strains tested when compared with VCZ. Döring and colleagues (2014) reported similar result with ITC in clinical strains obtained from paediatric patients. Indeed, it is important to highlight that our samples came from iron-rich sediment, which could be inducing resistance mechanisms against azoles as already observed in clinical and *in vivo* assays (Prasad et al., 2006; Craven et al., 2007; Zarember et al., 2009; Chowdhary et al., 2013; Hosogaya et al., 2013).

Hosogaya and colleagues (2013) showed a strong positive relation between iron concentrations and resistance to azoles in *Candida glabrata*. The authors showed that the protein Dap1 is indirectly involved in the ergosterol biosynthesis and their results suggested heme binding by Dap1 is crucial for Erg11

activity and ergosterol biosynthesis, thereby being required for azole tolerance. Similar relation between iron depletion and susceptibility to azoles in yeasts (Prasad et al., 2006; Craven et al., 2007) and the filamentous fungi *A. fumigatus* (Zarember et al., 2009) has been reported. Our results open to a discussion on the role of iron concentrations in natural habitats and resistance to azoles that certainly deserves special attention in future investigations.

Despite its high toxicity, AMB is still used against IPA (Kousha et al., 1999). Amphotericin B MICs commonly range between 0.25 and 2 µg/mL (Kazemi et al., 2013) with resistance breakpoint at MIC >2 µg/mL (CLSI, 2008). Although results with AMB were lower in our study (with MIC50 at 0.5 µg/mL), a resistant strain of *A. flavus* was detected and other strains had MICs at breakpoint value (*A. flavus* and *A. terreus*). The high MICs observed in *A. terreus* to AMB, was not surprising, since resistance to AMB has already been reported for this species (Sutton et al., 1999; Singh & Paterson, 2005). To our knowledge this is the first report of an AmB-resistant strain of *A. flavus* isolated directly from cave sediment. Resistance to AMB has already been documented for other *Aspergillus* species obtained from clinical samples (Blum et al., 2008; Van Der Linden et al., 2011; Hadrich et al., 2012).

Interestingly, the resistant strain was collected from the sediment of an aphotic site covered with numerous actinomycete colonies. As mentioned by Khoo et al. (1994), AMB was firstly obtained from filamentous bacteria (*Streptomyces nodosus*). It has been recently reported that other microorganisms inhabiting extreme environments also produce AMB as survival mechanism enhancing their ecological fitness in competing for substrate (Svahn et al., 2015). Additionally, Seo et al. (1999) induced resistance to AMB from a wild strain of *A. flavus* after several conidial transfers on AMB enriched media. This could be occurring naturally among wild strains in the environment. It is plausible to hypothesize that constant natural exposure to antifungal compounds produced by competitors (bacteria or fungi) induces natural selection of drug-resistant strains. Similar relation has been reported among cave isolated bacteria (Bhullar et al., 2012). The authors detected resistance to over fourteen antibiotics by bacteria isolated from a pristine cave in the United States and associated resistance with their pan genome and adaptive response to ecological interactions.

Concerning the investigation of resistance mechanisms, it has been widely considered that the main antifungal action of AMB derives from the binding between ergosterol and AMB altering membrane permeability and causing leackage of cytoplasmatic components (Ermishkin et al., 1976; Khoo et al., 1994; Johnson & Einstein 2007; Laniado-Laborín & Cabrales-Vargas, 2009). Thus, some authors have associated the main resistance mechanism to AMB with decrease of ergosterol content to diminish binding sites. Others pointed that ergosterol decrease is not the main resistance mechanism in *Aspergillus* spp. (Alcazar-Fuoli et al., 2006; Blum et al., 2008; Alcazar-Fuoli & Mellado, 2013) with resistance being more related with response to oxidative stress (Blum et al., 2013). Our results showed a substantial ergosterol increase in the AmB-R strain when exposed to the agent. It is important to highlight that ergosterol is involved in several relevant functions in fungi (e.g., hyphal growth, conidiation, protection) and its decrease has been classified as a secondary resistance mechanism (Laniado-Laborín & Cabrales-Vargas, 2009; Mesa-Arango et al., 2014). Furthermore, the resistant strain showed lower susceptibility to free radicals, probably due to the overexpression of the antioxidant system. Our group previously showed that AMB led to ROS formation and lipid peroxidation in yeast cells (*Cryptococcus gattii*) and this phenomenon strongly increased the enzymatic activities of the antioxidant system (Ferreira et al., 2013).

The data obtained in the enzymatic assay (PER and SOD) showed that SOD played major role in the resistance of *A. flavus* isolated from RM3 cave. Fungal AMB-resistance has been related with enzymatic activity against oxidative stress for some fungi (Laniado-Laborín & Cabrales-Vargas, 2009; Mesa-Arango et al., 2014). Blum et al. (2008) showed that AMB-resistance in *A. terreus* was highly related with catalase production. Okamoto et al. (2004) showed that oxidative damage induced by superoxide was the main activity of AMB against the yeast *C. albicans*. In the other hand, it seems that peroxidases did not play a crucial role in the resistance mechanism against AMB.

Caving is a common practice that has been increasing worldwide, causing various impacts on the subterranean biodiversity (Cigna, 1993; Souza-Silva, 2008; Lobo & Moretti, 2009; Souza-Silva & Ferreira, 2009; Kartalis et al., 2011; Taylor et al., 2013). The use of ground water and pieces of speleothems for curative purposes by local population should also be mentioned (Souza-Silva et al., 2011; Taylor et al., 2013) especially considering the impacts and risks posed by this activity. The transport of cave microorganisms and their contact with individuals of poor immunologic conditions enhance the chances of infection by drug-resistant strains. Despite being important components of cave biodiversity microbial communities have been poorly investigated in management plans and monitoring of subterranean ecosystems (Bhullar et al., 2012; Taylor et al., 2013; Vanderwolf et al., 2013). In the past decade, Brazilian governmental agencies have required more detailed microbiological inventories in caves to reduce chances of fungal outbreaks. Although the chances of developing pulmonary fungal infections by other species than *Histoplasma capsulatum* while caving are scarce, these opportunistic infections are emerging diseases and should be more thoroughly investigated, as well as their resistance mechanisms. The high resistance to AMB observed corroborates with the idea that antibiotic resistance might be a natural event, resulting from competition among soil organisms and their metabolites.

In conclusion, our findings broaden the knowledge concerning the action of AMB against *A. flavus* and the

mechanisms of resistance involved. We also speculate that interactions with environmental conditions (e.g., iron concentration) and between organisms lead to an adaptive response towards natural selection of drug resistant microorganisms in caves.

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