Evaluation of laccases and melanization in clinical and environmental *Cryptococcus neoformans* samples by non-denaturing PAGE

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The increased incidence of infections caused by the opportunistic pathogen *Cryptococcus neoformans*, which mainly affects immunocompromised patients but can also infect immunocompetent individuals, has needed additional studies on this micro-organism's pathogenicity and factors related to virulence, such as enzyme production, for a better understanding of the aetiology of cryptococcosis. The aim of this study was to verify the applicability of non-denaturing PAGE for analysis of laccases by quantification of the amount of melanin pigment produced by clinical and environmental strains of *C. neoformans*. After incubation of the gel with the substrate L-dopa, strains produced melanin spots of a bright brown to black colour. Quantification of these spots was performed by densitometry analysis and the amount of melanin produced was calculated and compared among the strains. All strains showed laccase activity. Serotype B strains showed a higher melanin intensity than serotype A strains. Over half of the clinical strains (56.2%) showed the lowest melanin intensities, suggesting that melanin production may not be the main virulence factor against host defence. The clinical strain ICB 88 revealed two melanin spots on the gel, indicating the presence of two laccase isoforms. The environmental strains showed the highest values of melanin intensity, which may be related to previous exposure to environmental stress conditions.

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INTRODUCTION

Cryptococcus neoformans is a cosmopolitan fungus and a major opportunistic pathogen in immunocompromised hosts, accounting for a significant proportion of AIDS-related infections (Pinner *et al.*, 1995; Casadevall & Perfect, 1998). Cryptococcosis is responsible for high morbidity and mortality rates among patients with AIDS (Kovacs *et al.*, 1985; Rozenbaum & Gonçalves, 1994; Kozel, 1995; Fernandes *et al.*, 2000). However, cryptococcosis also can occur among immunocompetent hosts (Kokturk *et al.*, 2005; Chen *et al.*, 2008). Due to its increasing incidence, additional studies on the pathogenesis of this micro-organism are necessary for a better understanding of the aetiological agent of cryptococcosis and its virulence factors.

C. neoformans strains have been grouped into three varieties: *C. neoformans* var. *grubii* (serotype A), *C. neoformans* var. *gattii* (serotypes B and C; now named *Cryptococcus gattii*) and *C. neoformans* var. *neoformans*

(serotypes D and AD). We have found *C. neoformans* var. *neoformans* and var. *grubii* in clinical material, but var. *grubii* is the predominant aetiological agent in 99% of cases of infections in AIDS patients and other immunocompromised individuals. *C. gattii* can be isolated from environmental samples, but *C. gattii* serotype B has also been found to cause disease in immunocompetent individuals (Mitchell & Perfect, 1995; Casadevall & Perfect, 1998; Steenbergen & Casadevall, 2003).

Among the possible virulence factors of *C. neoformans*, melanin is thought to be a major factor and is thought to be involved in adaptation to environmental stress conditions. It is synthesized by laccase enzymes, a group of multifunctional enzymes, in medium containing substrates such as L-dopa, and the colonies become brown or black as result of pigmentation of the cell (Williamson, 1994; Wang *et al.*, 1995; Williamson *et al.*, 1998; Hamilton & Gomez, 2002; Nosanchuk & Casadevall, 2003; Plonka & Grabacka, 2006).

In this study, we used non-denaturing electrophoresis to study melanization in *C. neoformans*. This technique of soluble fraction analysis of disrupted cells of *Cryptococcus* species has been used previously with satisfactory results (Ikeda *et al.*, 2002). Here, we aimed to evaluate and compare the laccase enzymes from clinical and environmental strains of *C. neoformans* through melanin pigment production using non-denaturing PAGE.

METHODS

Isolates. Thirty Brazilian strains (15 clinical and 15 environmental isolates) were analysed. The strains were from serotypes A and B (Table 1) and were obtained from the ICB/USP fungal collection (São Paulo, Brazil). The *C. neoformans* strain ATCC 90112 was used as a standard.

Inoculum preparation and culture. All strains were subcultured on Sabouraud agar (MicroMed) supplemented with 2% yeast extract (Difco) at 30 °C for 48 h. The strains were also inoculated into 150 ml Sabouraud broth (MicroMed) supplemented with 2% yeast extract and incubated at 30 °C for 48 h with shaking at 150 r.p.m.

Laccase assay. The strains were processed before laccase extraction according to the method of Wang *et al.* (1995) with some modifications. Briefly, after growth in Sabouraud broth supplemented with 2% yeast extract, *C. neoformans* cells were collected, washed in water and PBS buffer, and resuspended in 1 ml of 50 mM NaH₂PO₄, 0.5 M NaCl (pH 6.3) to which 150 µl of the protease inhibitor PMSF (10% solution; Fluka Biochemika) was added for each millilitre of cell suspension. The cells were maintained on ice and disrupted with glass beads (2 mm diameter; Sigma) by sonication at 40% amplitude for 5 min with a 9.0 s pulse on and a 0.2 s pulse off in a Sonics Vibra cell model VC 750 at a frequency of 20 kHz. The lysate was clarified by centrifugation at 2147 g for 15 min at 0 °C. The protein concentration was determined using the Bradford assay method (Bradford, 1976). Equal amounts of protein extract obtained from cells were separated by non-denaturing 7% PAGE without SDS. After electrophoresis, the gels were stained with 2 mM L-dopa in 0.1 M citric acid, 0.2 M Na₂HPO₄ (pH 6.0) for 6 h. The laccase present in the protein extract catalysed the production of melanin pigment, which was deposited in the gel at the position of the laccase protein. The gel was screened with a computing densitometer (program Kodak 3.5.4) to determine the amount of pigment. Laccase from *Rhus vernicifera* (Sigma) was used as control for laccase activity.

RESULTS AND DISCUSSION

All strains showed laccase enzyme activity, with bright brown to black spots after incubation with 2 mM L-dopa. Lanes 2–5 and lanes 7–11 in Fig. 1 show typical spots from clinical and environmental strains, respectively. Melanin pigment was also produced by *C. neoformans* strain ATCC 90112, whilst the laccase from *R. vernicifera* produced three spots (Fig. 1, lanes 14 and 15, respectively).

Quantification of the melanin spots was performed by densitometry with the Kodak 3.5.4 program, and the proportion of melanin was calculated and compared among all the strains, with the strain with the lowest intensity of melanin being assigned a value of 1 (Table 1).

Melanin production in *C. neoformans* has been related to virulence factors (Kwon-Chung *et al.*, 1982; Kwon-Chung & Rhodes, 1986), and melanization on medium containing diphenolic compounds has been proposed for the identification and study of yeasts (Shaw & Kapica, 1972; Wang *et al.*, 1995; Ikeda *et al.*, 2002; Garcia-Rivera *et al.*, 2005).

The compound L-dopa was chosen as the substrate for revelation of laccase enzyme activity on the gel because, among diphenolic compounds, it has been shown to reveal the enzyme activity clearly after non-denaturing electrophoresis (Ikeda & Jacobson, 1992). In addition, the use of citrate phosphate buffer (pH 6.0) to prepare the L-dopa solution, as proposed by Ikeda & Jacobson (1992), gave a relatively clear background against which to determine the activity of the *C. neoformans* laccases; these authors also recommend preparing the gel using ammonium persulfate as the polymerizer on the same day of use for a clear background, but in our study no difference was observed using a gel prepared 1 day earlier.

All of the strains evaluated showed laccase enzyme activity on the gel after non-denaturing electrophoresis, with results similar to those of other authors (Ikeda & Jacobson, 1992; Wang *et al.*, 1995; Ikeda *et al.*, 2002).

The mobility of the laccases from *C. neoformans* serotype B was slightly lower than that of the enzymes from serotype A and showed higher levels of melanin intensity. This difference in mobility has been observed previously by

Fig. 1. Melanin synthesis by laccase enzymes after non-denaturing PAGE. Melanin synthesis was carried out *in situ* in the gel by incubation with the substrate L-dopa. Lanes for clinical strains: 1, ICB 169; 2, ICB 89 RN; 3, ICB 189; 4, ICB 88 (two spots, labelled a and b); 5, RO2; 6, blank. Lanes for environmental strains: 7, Sul amb 3; 8, B 60; 9, Sul amb 1; 10, ICB 184; 11, B 52; 12, blank. Control lanes: 13, blank; 14, *C. neoformans* ATCC 90112; 15, *R. vernicifera* (three spots, labelled 1, 2 and 3).

Table 1. Spot intensities with and without melanin on gels and the proportion of melanin from clinical and environmental isolates and from the *C. neoformans* strain ATCC 90112

Clinical strains				Environmental strains			
Strain/serotype	Spot intensity*	Melanin intensity†	Ratio‡	Strain/serotype	Spot intensity*	Melanin intensity†	Ratio‡
Blank 1	145.787			Blank 4	138.000		
ICB 81/serotype A	172.903	27.116	8.12	Sul amb 3/serotype A	219.268	81.268	24.34
ICB 154/serotype A	187.234	41.447	12.42	B 60/serotype A	184.975	46.975	14.07
VPBet/serotype A	246.117	100.330	30.05	Sul amb 1/serotype A	219.558	81.558	24.43
ICB 95/serotype A	186.855	41.068	12.30	ICB 184/serotype B	243.045	105.045	31.47
HNR 41/serotype A	165.585	19.798	5.93	B 52/serotype A	187.928	49.928	14.96
Blank 2	133.965			Blank 5	134.600		
RO1/serotype B	232.818	98.853	29.61	B 51/serotype A	240.288	105.688	31.66
HNR 39/serotype A	139.029	5.064	1.52	B 79/serotype A	186.744	52.144	15.62
ICB 155/serotype A	154.368	20.402	6.11	BO 5/serotype A	226.639	92.039	27.57
CN 103/serotype A	203.238	69.273	20.75	BO 2/serotype A	198.858	64.258	19.25
ICB 23/serotype A	218.818	84.853	25.42	ICB 187/serotype A	137.938	3.338	1.00
Blank 3	122.018			Blank 6	120.266		
ICB 169/serotype A	136.880	14.862	4.45	ICB 178/serotype A	203.911	83.645	25.06
ICB 89 RN/serotype A	163.955	41.937	12.56	C 7/serotype A	243.238	122.972	36.84
ICB 189/serotype A	173.046	51.028	15.28	C 18/serotype A	204.308	84.042	25.18
ICB 88/serotype B	178.317	56.299	16.87	B 78/serotype A	152.317	32.051	9.60
ICB 88/serotype B	135.733	13.715	4.11	B 96/serotype A	127.410	7.144	2.14
RO2/serotype A	319.614	197.596	59.20	Blank 7	150.321		
				ATCC 90112/serotype A	193.845	43.524	13.04

*Calculated by the Kodak 3.5.4 program.

†Calculated by subtraction of the value of the blank spot from the value of the spot for each strain.

‡The relative proportion of melanin was calculated relative to the strain with the lowest melanin intensity (ICB 187), which was assigned a value of 1.

Ikeda & Jacobson (1992), but no correlation with melanin intensity was proposed in their paper.

Our results showed that the highest levels of melanin were found among strains from both origins, but that over half of the clinical strains of *C. neoformans* (56.2 %) produced the lowest melanin intensities. This observation suggests that melanin production may not be the main virulence factor against host defence, and that the fungus probably uses other virulence factors to establish infection, such as capsule synthesis and enzyme secretion (Kozel, 1995; Casadevall & Perfect, 1998; Blackstock *et al.*, 1999; Steenbergen & Casadevall, 2003).

The clinical strain ICB 88, which has been identified as *C. neoformans* var. *gattii*, produced two melanin spots on the gel, indicating the presence of two laccase isoforms. This has been described previously by Ikeda *et al.* (2002) for a strain of *Cryptococcus curvatus*. In our case, the major spot (Fig. 1, lane 4, spot a) had the lower mobility. Differential migration of laccase isoforms on gels is in accordance with the results of Ikeda & Jacobson (1992).

We found that the *C. neoformans* var. *gattii* strains produced spots of melanin with higher intensities than those from other strains of serotype A and that they showed the ability to synthesize more than one laccase

isoform. This production of increased amounts of melanin is in agreement with the results of Khan *et al.* (2007), who found that serotype B isolates produced more intense pigmentation and consequently were less susceptible to antifungal drugs such as amphotericin B, fluconazole, ketoconazole and voriconazole.

The environmental strains showed the greatest variation in range of melanin intensities, presenting both the lowest values of melanin intensity and most of the highest values, which may be a result of adaptation to environmental stress and could be related to previous exposure to UV light and ionizing radiation (Hamilton & Gomez, 2002; Nosanchuk & Casadevall, 2003; Plonka & Grabacka, 2006).

In conclusion, *C. neoformans* of different serotypes was able to synthesize laccases that could be detected on gels after non-denaturing electrophoresis and revealed by spot formation with the substrate L-dopa. We found that virulence could not be associated with the origin of the sample, i.e. clinical or environmental. Several clinically emerging *C. neoformans* var. *gattii* strains have the ability to synthesize more than one laccase and at high levels. This ability was observed on the gel and may be related to reduced susceptibility to antifungal drugs and probably high resistance of strains to treatment. Additional studies with a larger number of strains from different origins and

of different serotypes are necessary to establish the correlation between melanin intensities on gels and the virulence and resistance of *C. neoformans* strains.

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