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MOLECULAR CHARACTERIZATION OF ENVIRONMENTAL *Cryptococcus neoformans* ISOLATED IN VITORIA, ES, BRAZIL

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SUMMARY

Cryptococcus neoformans is the major cause of fungal meningitis, a potentially lethal mycosis. Bird excreta can be considered a significant environmental reservoir of this species in urban areas, thirty-three samples of pigeon excreta were collected within the city of Vitoria, Brazil. *Cryptococcus neoformans* was isolated and identified using standard biochemical assays in ten samples. PCR amplification with primer M13 and orotidine monophosphate pyrophosphorylase (*URA5*) gene-restriction fragment length polymorphism (RFLP) analysis discerned serotypes and genotypes within this species. All isolates were serotype A (*C. neoformans* var. *grubii*) and genotype VNI. The two alternative alleles **a** and α at the mating type locus were determined by PCR amplification and mating assays performed on V8 medium. All isolates were *MAT* α mating type but only 50% were able to mate *in vitro* with the opposite mating type *MAT* **a** tester strains (JEC20, KN99a and Bt63). This study adds information on the ecology and molecular characterization of *C. neoformans* in the Southeast region of Brazil.

KEYWORDS: *Cryptococcus neoformans*; Epidemiology; Mating types; Genotyping; Serotypes.

INTRODUCTION

The basidiomycetous yeasts of the *Cryptococcus neoformans* species complex (composed of *C. neoformans* var. *neoformans, C. neoformans* var. *grubii,* and *C. gattii*) are the etiological agents of cryptococcosis, an opportunistic systemic mycosis that usually manifests as meningoencephalitis in both humans and animals. Other organs such as lungs and skin can also be infected[4]. Cryptococcal infections are believed to be acquired by inhalation of airborne propagules from environmental source, assumed to be either by desiccated yeast or by basidiospore, deposited in the alveoli of the hosts[12].

Under the revised taxonomy, the species *C. neoformans* consists of two varieties: *C. neoformans* var. *grubii* (serotype A) and *C. neoformans* var. *neoformans* (serotype D), and the hybrid of var. *grubii* and var. *neoformans* (serotype AD), whereas the species *C. gattii* comprises the serotypes B and C[17]. The *Cryptococcus neoformans* complex has been divided into eight major molecular types by M13 fingerprinting, *URA5*-RFLP and AFLP analysis[23,24]. These include VNI and VNII (*C. neoformans* var. *grubii*); VNIII (AD hybrid); VNIV (*C. neoformans* var. *neoformans*); VGI, VGII, VGIII and VGIV (*C. gattii*).

The distribution of serotypes around the world show that clinical and environmental isolates serotype A is present in a widespread distribution[4], whereas serotypes B and C were mostly limited to tropical and subtropical regions[31]. However, the ecological niche of *C. gattii* has expanded due to discoveries of the yeast in temperate climate zone[8,13]. The occurrence of serotype D is scarcely reported except in some European countries[7] and areas of the United States[33] and it was recently isolated in Southern and South Eastern regions of Brazil from Eucalypt trees[28]. In Brazil, the epidemiology of serotype A in the southern and southeastern regions reproduces the picture observed worldwide. On the contrary, serotype B is the most frequent agent of cryptococcosis in the northeastern region[23,27]. While *C. gattii* is found to be associated with trees, *C. neoformans* is mostly recovered from bird excreta[4].

Two mating types α and **a** are recognized in *C. neoformans*[14]. Over 95% of all clinical and environmental isolates of *C. neoformans* are *MAT*α serotype A isolates (Aα)[11,37,38] and with worldwide distribution. This bias in mating type ratios has been postulated to be caused by wild-type haploid *MAT*α cells of *C. neoformans* that could develop a hyphal phase under appropriate conditions, producing basidia with viable basidiospores[16,37]. Because of their small size, basidiospores are more effectively dispersed than the encapsulated vegetative yeast cells. Thus, haploid fruiting of *MAT*α strains may explain the predominance of this mating type among environmental and clinical isolates.

The purposes of this study were to evaluate, by molecular methods, the serotypes, mating types and fertility of *C. neoformans* environmental strains collected in Vitoria, Espirito Santo State, Brazil and to determine the extent of genetic diversity among them.

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MATERIALS AND METHODS

Sampling: Thirty-three samples of pigeon excreta were collected from outside and attic of public buildings, dockside warehouse, monuments and squares within the city of Vitoria, Brazil. A total of eight surveyed sites were selected because they were highly contaminated with pigeon excreta and have a significant flow of residents and tourists. The samples were treated as described by STAIB *et al.*[32]. after suspension of approximately 5 g of each pigeon excreta sample in sterile saline with chloramphenicol (0.05 g/L), 100 µL aliquots were spread on 10 plates with niger seed agar with 0.05 g/L chloramphenicol and 0.1 g/L biphenil. The plates were incubated at 27 ºC for two weeks. Dark brown colonies were confirmed as *C. neoformans* according to morphological characteristics and standard biochemical assays.

DNA isolation: Genomic DNA was extracted based on the method described by DEL POETA *et al.*[6]. Briefly one loop of the *C. neoformans* grown on YEPD agar (1% yeast extract, 2% peptone, 2% dextrose and 2% agar) were suspended in 0.2 mL of 0.45 mm glass beads and 0.5 mL lysis buffer (50mM Tris, pH7.5; 20mM EDTA, pH8.0; 200mM NaCl; 2% triton; 1% SDS). The cells were disrupted by vigorous vortex, and then purified nucleic acids were extracted three times with phenol-chloroform-isoamyl alcohol (24:24:1). The aqueous phase was transferred to a fresh tube and genomic DNA was precipitated with absolute ethanol. Precipitated DNA was collected, resuspended in 0.5 mL TE buffer (10mM Tri-HCl, 1mM sodium EDTA, pH 8.0) and stored at -20 ºC.

PCR-fingerprinting: Oligonucleotides of the minisatellite-specific core sequence of the wild-type phage M13 (5'-GAGGGTGGCGGTTCT-3') were used as single primers for PCR amplification, according to MEYER *et al*.[23]. PCR products were separated by electrophoresis in 1.4% agarose gel in 1XTBE buffer stained with ethidium bromide at 0.5 ng mL^{-1} and visualized under UV light. Reference isolates were used as comparison namely ATCC 34871 (serotype A)[29], RV 45981 (serotype D)[25], NIH312 (serotype C)[9] and B4546 (serotype B)[9].

PCR-RFLP: *URA5* gene of each strain was amplified and digested as previously described[24]. The digested amplicons were run on 3% agarose gel against the reference strains [WM148 (VNI), WM626 (VNII), WM628 (VNIII), WM629 (VNIV), WM179 (VGI), WM178 (VGII), WM175 (VGIII), WM779 (VGIV)] to determine their molecular types.

Determination of mating type by PCR: The mating types were determined by PCR with specific primer to the *STE20*α and *STE20***a** gene sequences, developed by LENGELER *at al.*[19]. The primers were *STE20*α: JOHE 7264/KBL 5'AGC TGA TGC TGT GGA TTG AAT AC 3', JOHE 7266/KBL 5'TGC AAT CAC AGC ACC TTA CAT AG 3' and JOHE 7267/KBL 5'ATA GGC TGG TGC TGT GAA TTA AG 3', JOHE 7269/KBL 5'TGC AGT CAC AGC ACC TTC TAT AC 3' for *STE20***a**. Amplification reactions were performed as described by the authors and approximately 1Kb fragment was amplified from the mating type locus. The PCR amplicons were electrophoresed on 0.7% agarose gel in 1XTBE buffer at 100 V and then, stained in a solution of ethidium bromide at 0.5 ng mL^{-1}. The gels were visualized by UV transilumination and photographed. Two positive controls were used: JEC 21 (serotype D, *MAT*α) and JEC 20 (serotype D, *MAT* **a**)[20,26].

Mating experiments: Strains were pregrown on YEPD agar for two days, and a little amount of cells was removed and patched onto solid mating medium (5% V8 juice, 3 mM KH_2PO_4, 4% agar) pH 5.0 and pH 7.0, either alone or mixed with the mating type **a** strains. All the plates were incubated in darkness at 25 ºC for up to four weeks[15]. The ability to mate was tested against the serotype A mating type **a** strains KN99a and Bt63 and the serotype D mating type **a** JEC20[21,26]. The ability to undergo same-sex mating were tested with the serotype A mating type α strains KN99α and the serotype D mating type α JEC21. The haploid fruiting ability was tested by incubation of each strain alone. Plates were examined regularly for evidence of filamentation and basidiospore chains indicating mating reaction.

RESULTS

Sampling: Cryptococcus neoformans was isolated from 10/33 samples (30%) of positive pigeon excreta (Table 1). All of them were from public areas with great flow of people. All isolates were obtained from old, dry and withered pigeon dropping, not fresh, and the yeast density in samples was above 1 x 10^3 CFU/g. Only samples obtained from places shielded from direct sunlight and rain were positive for *Cryptococcus neoformans*.

PCR fingerprinting: The minisatellite-specific primer M13 was able to generate individual strain-specific DNA polymorphism, allowing the easy differentiation among the two serotypes of *C. neoformans* (A and D) as well as serotypes B/C of *C. gattii* (Fig. 1). PCR fingerprinting profile revealed a high level of homogeneity among strains and gave identical patterns for serotype A when compared with reference strain ATCC 34871. The *URA5* RFLP confirmed all the strains to be VNI molecular type (Fig. 2).

Determination of mating type: Studies revealed that all environmental *Cryptococcus neoformans* serotype A strains were *MAT*α mating type as determined by PCR amplification of the *STE20*α gene sequence, producing an expected DNA fragment of 1 Kb for each strain. The primers STE20**a** did not amplify any DNA fragment from serotype A strains indicating that all strains A had only *MAT*α alleles (Fig. 3).

Mating experiments: Mating studies showed that 50% of 10 isolates were able to mate with their opposite mating type *MAT**a*** tester isolates (JEC20, KN99a and Bt63). It confirmed the *MAT*α mating type detected by PCR, since none of the isolates mated with *MAT*α mating type tester strains JEC21 and H99, and, thus, no same-sex mating. We graded the mating typing experiment results into three categories: sterile, fertile and robustly fertile and observed that only strain number 5 fit the third category (Fig. 4). Interestingly, this strain was not able to grow at the temperature of 37 ºC (data not shown).

DISCUSSION

Our results support the molecular application for typing on serotypes of *C. neoformans* as shown in a previous study[23]. MEYER *et al.*[24] showed that the majority of the environmental isolates (73%) in Latin America were VNI. The overview of clinical and environmental genotypes distribution suggests, however, heterogeneity of VNI distribution, with the highest number of isolates (93%) in Peru and Guatemala and lowest number (42%) in Chile. Brazil accounted with 82% of VNI isolates in this study. The overall results show the worldwide environmental

Table 1
Isolation of *Cryptococcus neoformans* according to habitat

Local	Samples	Sheltered	Isolation
Public building	P1A1	No	Negative
	P1A2	Yes	**Positive**
	P1A3	Yes	**Positive**
	P1A4	No	Negative
	P1A5	No	Negative
	P1A 6	Yes	**Positive**
Public building	P2A1	No	Negative
	P2A 2	No	Negative
	P2A3	Yes	Negative
Public building	P3A1	Yes	**Positive**
	P3A2	Yes	Negative
	P3A3	Yes	**Positive**
	P3A4	No	Negative
	P3A5	No	Negative
Public building	P4A1	No	Negative
	P4A2	Yes	**Positive**
	P4A3	Yes	Negative
	P4A4	No	Negative
Squares	P5A1	No	Negative
	P5A2	No	Negative
	P5A3	No	Negative
	P5A4	No	Negative
Monuments	P6A1	No	Negative
	P6A2	No	Negative
	P6A3	No	Negative
	P6A4	Yes	**Positive**
Dockside warehouse	P7A1	Yes	Negative
	P7A2	No	Negative
	P7A3	Yes	**Positive**
	P7A4	Yes	Negative
Dockside warehouse	P8A1	Yes	**Positive**
	P8A2	Yes	**Positive**
	P8A3	Yes	Negative

predominance of serotype A (*C. neoformans* var. *grubii*) with an identical or similar PCR fingerprinting and RAPD profiles, indicating a conserved molecular pattern in this serotype.

This method does not only substitute the immunological serotyping in *C. neoformans*, but also shows a high discriminatory power. Genotyping of environmental isolates with primer M13 allowed the differentiation between the two varieties types of *C. neoformans* according to MEYER *et al*.[23]. However, a problem occurs with primer M13: it does not differentiate serotypes D and AD[24]. In this study we did not detect any hybrid strains, molecular type VNIII, confirmed with *URA5* RFLP (Fig. 2).

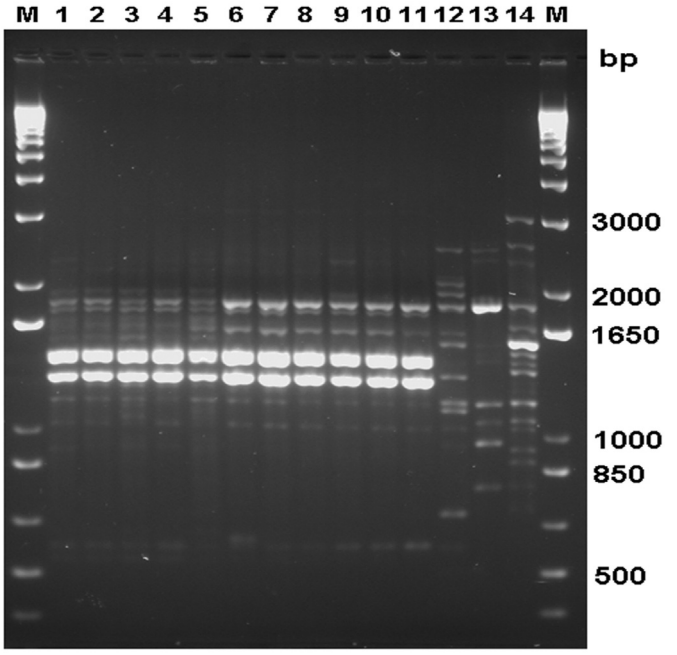

Fig. 1 - The PCR fingerprints using the M13 primer reveal the similar pattern between *C. neoformans* in this study and the reference serotype A strain (ATCC 34871). Lines 1-10: environmental *C. neoformans* strains; lines 11-14: reference strains: serotypes A, B, C and D. M: molecular marker (1Kb+ DNA ladder, Invitrogen®)

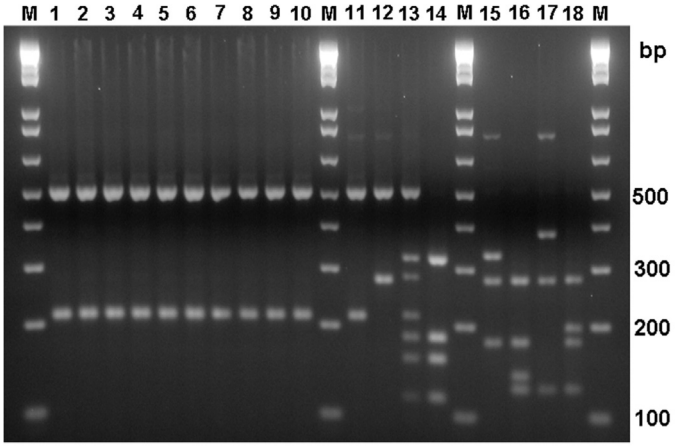

Fig. 2 - The *URA5*-RFLP of *C. neoformans*. Lines 1-10: environmental *C. neofomans* strains from this study; lines 11-14: reference strains VNI, VNII, VNIII, VNIV; lines 15-18: *C. gattii* reference strains VGI, VGII, VGIII, VGIV. M: molecular marker (1Kb+ DNA ladder, Invitrogen®). All strains collected in this study belong to molecular type VNI.

Less attention has been paid to environmental sources of fungi, despite its epidemiological relevance. The demonstration of geographical clustering of isolates based on environmental samples may be more reliable than clinical ones, since the distribution of profiles according to environmental isolates would reflect autochthonous infections. Indeed,

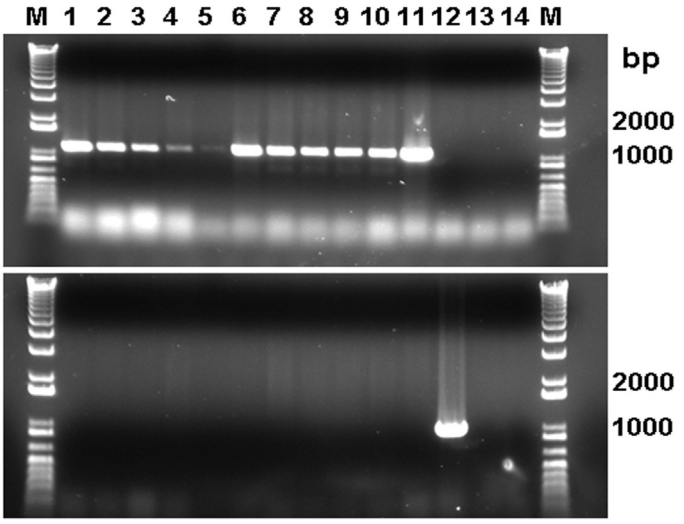

Fig. 3 - Amplification with primer specific for mating typing loci: (A) with primer JOHE 7264/KBL and 7266/KBL for *STE20*α gene; (B) with primer JOHE 7267/KBL and 7269/KBL for *STE20***a** gene**.** Lines 1 to 10: environmental strains from this study. Line 11: reference strain JEC 21 (serotype D, *MAT* α) and line 12: reference strain JEC 20 (serotype D, *MAT* **a**). M: molecular marker (1Kb+ DNA ladder, Invitrogen®). All strains from this study belonged to mating type α. Lines 13 and 14: Internal control with *Candida albicans*.

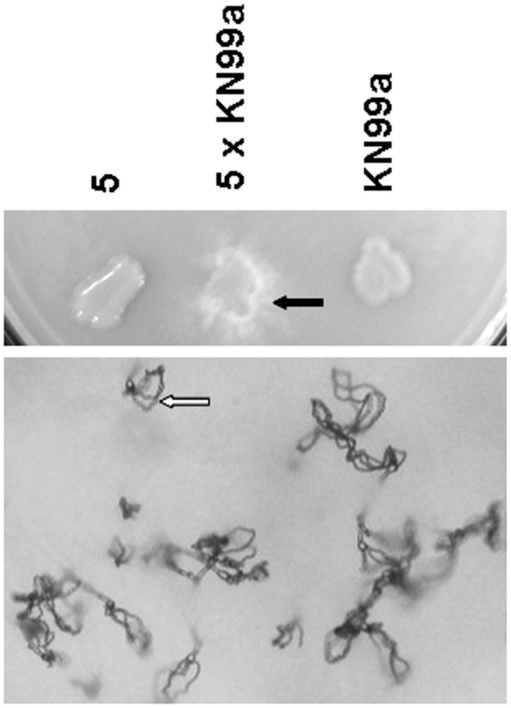

Fig. 4 - Example of mating typing reaction between strain number 5 and its opposite mating type *MAT***a** tester isolate KN99a. The black and white arrow represents filamentation and basidiospores chains on V8 medium, respectively.

GARCIA-HERMOSO *et al.*[10] showed that the distribution of clinical isolates from African patients diagnosed with cryptococcosis in France was significantly different from that of clinical isolates recovered from the European patients, suggesting that African patients might have been infected in their native countries but not in France. TINTELNOT *et al.*[34] also studying patients with cryptococosis among European residents, at the beginning of the illness, informed that 22% of these patients originally came from Asia.

The city of Vitoria, Espirito Santo State, built on an area of 45 km^2 in area consists of colonial buildings, ports and beaches. The climate is tropical and humid, with an average maximum monthly temperature of 30.4 °C (86.72 °F) and minimum of 24 °C (75.2 °F), and a heavy rainy season mainly in the months of October to January. It lies on the Southeast part of the Brazilian coast and this study revealed a comparable result as environmental surveys in the same geographic and climatic area, namely Rio de Janeiro[18] and Sao Paulo[30], where *C. neoformans* serotype A was predominantly recovered from pigeon excreta.

Only a few Brazilian studies have investigated the molecular types of environmental *Cryptococcus*[1,2,3,22,24,28,35]. The distribution of molecular types using the specific primer M13 shows the predominance of VNI (serotype A) in surveyed regions. It is worthwhile to note the presence of VNIV (serotype D) in eucalypt trees in the deep south of Brazil, a subtropical region[2,28], and of VGI (*C. gattii* serotype B) in excreta of psittaciformes in the same region[1]. Study of clinical isolates in São Paulo showed that all of them were serotype A and the majority belonged to the molecular type VNI[22].

The sexual cycle of *C. neoformans* involves fusion of **a** and α cells determined by two alleles within the single *MAT* locus, designated *MAT*α or *MAT***a**[14]. In this study, all the environmental isolates produced amplicons only of STE20α (Fig. 3) and, it was observed that the PCR method was more successful in determining mating type alleles than mating experiments as previously shown[21,33,34], since mating in the laboratory with *MAT**a*** tester strains (JEC20, KN99a and Bt63) identified only 50% of the *MAT*α strains. Among them, same-sex mating of the isolates was ruled out because none of the *MAT*α strains mated with opposite mating type *MAT*α taster strains (JEC21 and H99).

No *MAT**a*** strain was recovered in this study. This emphasizes the sexual reproduction by opposite mating type which does not appear to be dominant in *C. neoformans* var. *grubii*. An imbalanced ratio of **a**/α spores generation is frequently observed and the rare *MAT**a*** serotype A isolates (A**a**) accounted with only 3 of >2,000 isolates examined[21] and they were discovered confined in specific geographical regions of Africa, such as Tanzania[19], Botswana[21], and Italy[36].

This study shows the environmental predominance of *Cryptococcus neoformans* serotype A, molecular type VNI and mating-type α in the city of Vitoria and adds information on the ecology and molecular characterization of *C. neoformans* in the Southeast region of Brazil.

RESUMO

Caracterização molecular de cepas ambientais de *Cryptococcus neoformans* isoladas em Vitória, ES, Brasil

O "complexo *Cryptococcus neoformans*" é constituído por *C. neoformans* var. *neoformans, C. neoformans* var. *grubii,* e *C. gattii*. Trinta por cento de amostras de excrementos de pombos coletados dentro da cidade de Vitória, Brasil, foram positivas para *Cryptococcus neoformans*, espécie identificada por testes bioquímicos convencionais. Amplificação

por PCR com primer M13 e análise por orotidine monophosphate pyrophosphorylase (*URA5*) gene-"restriction fragment length polymorphism" (RFLP) distinguiram sorotipos e genotipos dentro desta espécie. Todos os isolados ambientais foram sorotipo A (*C. neoformans* var. *grubii*) e genotipo VNI. Os dois alelos alternativos **a** e α do locus "mating type" foram determinados por PCR e por testes de "mating" em meio V8. Todos os isolados foram "mating type" tipo *MAT* α mas somente 50% foram capazes de conjugar *in vitro* com cepas *MAT* **a**, de "mating type" oposto (JEC20, KN99a e Bt63). Este estudo adiciona informações sobre a ecologia e caracterização molecular de cepas ambientais de *C. neoformans,* isoladas na região sudeste do Brasil.

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