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SEROTYPE, MATING TYPE AND PLOIDY OF *Cryptococcus neoformans* STRAINS ISOLATED FROM PATIENTS IN BRAZIL

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SUMMARY

Serotype, mating type and ploidy of 84 strains of *Cryptococcus neoformans* isolated from 61 AIDS and 23 non-AIDS patients admitted in a tertiary teaching hospital in São Paulo, Brazil were examined. Among 61 strains isolated from AIDS patients, 60 strains were var. *grubii* (serotype A). Only one strain was var. *gattii* (serotype B). No var. *neoformans* (serotype D) was found. Among 23 strains isolated from non-AIDS patients, 15 were var. *grubii* (serotype A) and the remaining 8 were var. *gattii*, all of which were serotype B. Seventy-three of the 75 serotype A strains were the heterothallic α type (MATα) and the remaining 2 were untypable (asexual). Most of the MATα strains (69/73) were haploid and the remaining 4 strains were diploid. Similarly, both of the 2 asexual strains among the 75 serotype A strains were haploid. There were no *α*-mating type (MAT*a*) strains among the 84 isolates. All of the 8 var. *gattii* strains were serotype B and haploid. Among a total of 84 strains tested, neither serotype AD nor serotype D were found. Neither triploid nor tetraploid were found. These results suggest that the serological, sexual and ploidy characteristics in *C. neoformans* strains isolated from AIDS patients in São Paulo were rather simple, whereas strains isolated from non-AIDS patients presented serotype A and B with predominance of serotype A.

KEYWORDS: AIDS; *Cryptococcus neoformans*; Mating type; Ploidy; Serotype

INTRODUCTION

Cryptococcus neoformans, the anamorph of *Filobasidiella neoformans,* exists worldwide in nature, such as in pigeon droppings and *Eucalyptus* trees[3,17]. It causes the most serious deep-seated mycoses among the basidiomycetous fungi, and is one of the most life-threatening pathogens in AIDS patients[3]. Two mating loci with four different mating alleles are common among basidiomycetous fungi. *C. neoformans,* however, has only one mating locus with two mating type, i.e., alpha (MATα) and *a* (MAT*a*). The α-mating type strains have been shown to be more virulent than the *α*-mating types[12]. *C. neoformans* has five serotypes (A, B, C, D and AD), and is subdivided into the three varieties, i.e. var *grubii* (serotype A), var. *neoformans* (serotypes D) and var. *gattii* (serotypes B and C)[6]. PCR analysis with primers specific for genes in the MAT*a* or MATα mating-type loci revealed that serotype AD strains are heterozygous for the mating locus. Serotype AD strains of *C. neoformans* are unusual aneuploid or diploid strains that result from matings between serotype A and D strains[16]. Var. *grubii* is geographically distributed throughout the world, while var. *gattii* is most frequently found in tropical and subtropical regions[17]. Var. *neoformans* is isolated rather frequently in Europe and South America[17]. Thus, it is of interest to determine the varieties and serotypes of *C. neoformans* isolates from patients in Brazil where all the varieties are endemic[2,14,15,18,20,21,22].

Mating type and ploidy are also important for understanding the ecology and virulence of this fungus[1,12]. The serotype and mating type of clinical and environmental isolates *of C. neoformans* have been studied rather well, but only a few studies on ploidy have been performed with a large number of environmental and clinical isolates[8,24]. In the present study we report on the serotype, the mating type as well as ploidy of 84 *C. neoformans* strains isolated from AIDS and other patients living in São Paulo and surrounding areas.

MATERIAL AND METHODS

Test organisms: Eighty-four *C. neoformans* strains were isolated from patients with cryptococcosis who were hospitalized from 1996 to 2000 in Hospital das Clínicas (a 2200 bed tertiary care teaching Hospital, School of Medicine, University of São Paulo - SP, Brazil) were included in the study. These are listed in Tables 1 and 2, together with strain designations and with IFM (Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University) number.

The majority of the isolates were from cerebral spinal fluid and blood respectively. The strains were identified by colonial morphology (demonstration of capsule, spherical shape, growth at 37 ºC), conventional biochemichal test[5] and/or using YBC Card Vitek System (Bio Mérieux,

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Table 1
Ploidy, serotype and mating type of *C. neoformans* isolated from AIDS patients

Strain	IFM No.*	Serotype	Mating type	DNA content	Ploidy	Strain	IFM No.*	Serotype	Mating type	DNA content	Ploidy
48	51611	A	α	1.00	H	582	51652	A	UT[a]	1.02	H
54	51612	A	α	1.18	H	695	51655	A	α	1.00	H
55	51613	A	α	0.94	H	708	51657	A	α	1.76	D
56	51614	A	α	0.98	H	742	51661	A	α	0.84	H
57	51615	A	α	1.02	H	754	51662	A	α	0.88	H
58	51616	A	α	1.02	H	772	51663	A	α	0.80	H
65	51617	A	α	0.86	H	791	51664	A	α	0.98	H
124	51618	A	α	1.00	H	795	51653	A	α	0.86	H
181	51619	A	α	1.00	H	807	51666	A	α	1.06	H
204	51620	A	α	0.98	H	808	51667	A	α	1.00	H
228	51621	A	α	0.90	H	838	51668	A	α	0.84	H
253	51623	A	α	1.00	H	853	51670	A	α	0.96	H
298	51624	A	α	0.98	H	861	51671	A	α	0.94	H
305	51622	A	α	0.94	H	863	51672	A	α	0.82	H
349	51625	A	α	0.98	H	884	51674	A	α	0.98	H
350	51626	A	α	0.90	H	890	51675	A	α	0.92	H
352	51627	A	α	1.20	H	909	51677	A	α	1.10	H
367	51628	A	α	0.92	H	917	51678	A	α	1.20	H
369	51630	A	α	0.92	H	926	51680	A	α	0.82	H
370	51631	A	α	1.00	H	946	51682	A	α	1.00	H
387	51632	A	α	0.94	H	957	51685	A	α	0.90	H
403	51634	A	α	0.84	H	961	51684	A	α	0.90	H
410	51635	A	α	0.82	H	980	51694	A	α	0.88	H
412	51636	A	α	0.80	H	981	51683	A	α	0.86	H
416	51641	A	α	0.84	H	982	51686	A	α	0.86	H
419	51637	B	UT[a]	0.88	H	1014	51687	A	α	1.08	H
423	51638	A	α	0.92	H	1028	51689	A	α	1.96	D
426	51639	A	α	0.98	H	1039	51690	A	α	1.00	H
446	51642	A	α	1.98	D	1049	51691	A	α	0.86	H
462	51643	A	α	2.14	D	J.C	51692	A	α	1.00	H
549	51650	A	UT[a]	1.12	H						

* Strain number of the Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University; a = Untypable mating type.

Table 2
Ploidy, serotype and mating type of *C. neoformans* isolated from patients other than AIDS

Strain	IFM No.*	Serotype	Mating type	DNA content	Ploidy	Strain	IFM No.*	Serotype	Mating type	DNA content	Ploidy
368	51629	B	UT	0.88	H	734	51658	A	α	1.10	H
393	51633	A	α	0.90	H	738	51659	A	α	0.84	H
439	51640	A	α	1.12	H	741	51660	B	UT[a]	0.86	H
477	51644	A	α	0.90	H	801	51665	A	α	0.82	H
484	51645	B	UT[a]	0.98	H	854	51669	A	α	1.02	H
489	51647	A	α	0.84	H	878	51673	A	α	0.88	H
491	51648	A	α	0.92	H	891	51676	B	UT[a]	0.92	H
509	51649	A	α	1.04	H	918	51679	A	α	0.90	H
571	51651	B	UT[a]	1.02	H	927	51681	A	α	0.94	H
643	51654	B	UT[a]	1.06	H	1024	51688	B	UT[a]	0.88	H
694	51646	A	α	0.96	H	J.N	51693	B	UT[a]	0.86	H
696	51656	A	α	0.84	H						

* Strain number of the Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University; a = Untypable mating type

USA). In addition, their phenoloxidase activity (melanin production) was tested with the DOPA agar medium[4].

All of the strains were maintained on Sabouraud glucose agar slants. *C. neoformans* strains giving blue color in canavanine-glycine-bromothymol blue test[13] were judged as var. *gattii*. Those showing yellow color were judged as variety group of var. *neoformans* and var. *grubii*. The group was further subdivided by serotypes into two varieties. Those with serotype D was judged as var. *neoformans*. Those with serotype A was judged as var. *grubii*.

Serotyping: The slide agglutination tests with 6 factor sera[9] were

performed using the Crypto Check Kit (Iatron Laboratories) as previously described by TAKEO *et al.*, 1993[24].

Mating tests: The method was essentially the same as described in KWON-CHUNG & BENNETT (1978)[10]. The strains IFM 5844 (= NIH B-3501) and IFM 5845 (= NIH B-3502) of *Filobasidiella neoformans* var. *neoformans* were used as the tester strain α and *a*, respectively. Small amount of yeast cells on a loop from 1-2, day-old cultures on potato dextrose agar slant at 25 °C was mixed with similar amount of cells of the α or α tester strain in distilled water and incubated for 7 weeks on hay cube agar at 25 °C. To check homothalism of each strain, the above incubation was also done without mixing the tester strains. The existence of filamentous cells with clamp connections, basidia, and basidiospores was checked at one week interval until 7 weeks under a stereoscope Nikon SZ-PT, or a light microscope Olympus BS2. Only those strains having the above set of the sexual structures were judged as positive.

Analysis of cellular DNA: The methods were used as described previously by TAKEO *et al*., 1995[23]. Briefly, cellular DNA was stained quantitatively with 5 μg ml^{-1} propidium iodide and RNA was digested using 0.5 mg ml^{-1} RNase from bovine pancreas (Sigma type 1-AS). Then, DNA content was examined in each cell with regard to cell morphology using a laser-scanning-cytometer Olympus model LSC 101 or a fluorescence microscope Olympus model BS2 equipped with a photomultiplier[19].

RESULTS

Strains isolated from AIDS patients: Among a total of 61 strains isolated from AIDS patients, 60 strains did not grow efficiently and did not change the light green color of the agar medium to cobalt blue when inoculated onto CGB agar medium. These strains should belong to either var. *neoformans* or var. *grubii*.

The results of serotyping, mating type determination and ploidy assay of a total of 61 strains are shown in Table 1. Among the 61 strains, all except one were serotyped as A. One strain was serotype B. No strains possessed serotype C, D or AD. Thus, strains isolated from AIDS patients were essentially var. *grubii*.

The sexual characteristics of the isolates were determined according to the emergence of hyphae with clamp connections, basidia and basidiospores when mated with the *a* and α tester strains in a sporulation medium. Among the 61 strains tested, 58 (94%) were of the heterothallic MATα type and 3 gave no distinctive sexual responses. Neither the heterothallic MAT*a* type nor homothallic MAT*a*/α type were found. Fifty four (90%) of the 58 MATα strains were haploid and 4 were diploid. All the 3 asexual strains (two serotype A and one serotype B) were haploid.

Morphologically, it was also noted that the cells and nuclei of the haploid strains were similarly small in size. On the contrary, the cellular and nuclear sizes of the diploid strains were distinctly larger than those of the haploid strains. Neither triploid nor tetraploid strains were observed.

Strains isolated from non-AIDS patients: Fifteen strains were found to be var. *grubii* (serotype A). These were all MATα and haploid. Eight var. *gattii* strains were all serotype B. Consistent with the results of color development in the CGB test, none of the strains from var. *gattii* were of the A, D or AD serotypes. As to mating type, these did not respond well to both of the tester strains. They were all haploid.

DISCUSSION

The different geographical distribution of the three varieties of *C. neoformans* have aroused attention to a possible relationship between varieties of causative strains and countries. Interestingly, a number of epidemiological studies have demonstrated that almost all cryptococcal infections in AIDS patients are due to var. *neoformans* or var. *grubii* (serotype A or D) strains, even in the var. *gattii*-epidemic areas[2,10,11,15,20]. Concerning *C. neoformans* strains isolated from cryptococcosis patients in Brazil, var. *neoformans* (serotypes A, actually *C. grubii*) and var. *gattii* (serotype B) were the common causative agents of cryptococcosis and the number of cases increased after the advent of AIDS[2,14,21,22]. As clearly shown in Tables 1 and 2, our results support the above observations, and furthermore demonstrate that serotype A is predominant in the *C. neoformans* isolates from AIDS patients in São Paulo and surrounding areas.

The mating types of *C. neoformans* isolates are important for understanding their ecology and virulence. The α-mating type strains are much more prevalent than α-mating type strains in environmental and clinical isolates. MATα strains have been shown to develop extensive hyphae, without mating, and to produce viable basidiospores under appropriate conditions. This ability, found only in the MATα strains, explains the mating type ratios in environmental and clinical isolates[1]. There were no *a*-mating type strains among the 84 strains examined in the present study. In our study, 2 of the 84 isolates were asexual. In the literature, the incidence of asexual (untypable) strains was about 10%, except for var. *gattii* strains. Var. *gattii* strains are notorious for difficulty in obtaining the sexual stages[7]. Here 8 *gattii* strains were obtained from 8 non-AIDS patients, all of them did not show mating ability, although they were all haploid. Self-fertile MAT*a*/α strains were not found in our 84 isolates. KWON-CHUNG *et al.* reported that the MATα type was more virulent than the opposite *a*-mating type[10,12]. The predominance of the MATα strains observed in the present study is in accordance with their findings, since the strains examined were isolated from patients.

Strains, isolated from São Paulo and surrounding areas, were mostly haploid. This is in good agreement with the well known fact that most *C. neoformans* strains show mating ability as heterothallic MATα. We found no homothallic MAT*a*/α and asexual diploid strains, but 4 heterothallic MATα diploid strains. As to ploidy determination with a large number of environmental and clinical isolates, there have been reported only on our two previous papers. The first paper deals with the strains isolated from non-AIDS patients and environment essentially in Japan[24] and the other, from AIDS patients in Thailand[8]. Thus, the strains examined in this and in the former two papers were isolated from three different geographical regions. However, heterothallic MATα diploid strains are consistently found at relatively high incidence of more than 5%. Thus, the existence of heterothallic MATα diploid strains can be regarded as a cellular characteristic of *C. neoformans*.

RESUMO

Sorotipos, "mating type" e ploidia de amostras de *C. neoformans* isoladas de pacientes no Brasil

Foram estudados os sorotipos, "mating type" e ploidia de 84 amostras

de *C. neoformans* isoladas de 61 pacientes com AIDS e 23 não-AIDS em São Paulo. Das amostras isoladas de pacientes com AIDS, 60 foram identificadas como var. *grubii* (sorotipo A) e 1 como var. *gattii* (sorotipo B). Não houve isolamento do sorotipo D. Entre as amostras isoladas, de pacientes não-AIDS, 15 foram de var. *grubii* (sorotipo A) e as 8 restantes de var. *gattii,* todos do sorotipo B. Setenta e três dos 75 sorotipos A foram identificadas como cepas heterotálicas do fenótipo α (MATα) e as 2 remanescentes não-tipáveis (assexuada), eram haplóides. A maioria das cepas MATα (69/73) era haplóide sendo 4 diplóide. Não houve o isolamento de fenótipo *a* (MAT*a*) entre as 84 cepas analisadas. Todas as 11 amostras de var. *gattii* eram do sorotipo B e haplóides. Não foram observados os sorotipos AD e C, nem células triplóides ou tetraplóides entre as 84 amostras estudadas. Os resultados sugerem, que as características sorológicas, sexuais e de ploidia de *C. neoformans,* isoladas de pacientes com AIDS em São Paulo, são particularmente simples, a maioria do sorotipo A, enquanto que nos pacientes não-AIDS foram observados tanto os sorotipos A quanto o B.

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