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Correlation of antifungal susceptibility and molecular type within the *Cryptococcus neoformans/C. gattii* species complex

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Members of the *Cryptococcus neoformans/C. gattii* species complex are grouped into eight molecular types, differing in their epidemiology, disease severity and geographic range. Recent *in vitro* antifungal susceptibility studies of isolates of the complex revealed contradictory results. The objective of the present study was to assess if this variation is random or correlates with different molecular types by testing the *in vitro* antifungal susceptibility of 18 *C. neoformans* (VNI), 11 *C. gattii* (VGI) and 38 *C. gattii* (VGII) strains from Brazil to eight antifungal drugs using the CLSI microdilution method. We herein report that the molecular type VGII is the least susceptible genotype, followed by VGI and VNI. This indicates a clear correlation between antifungal susceptibilities and genotypes of the causative cryptococcosis agents, emphasizing the importance of determining the molecular type as part of the clinical diagnostic process to enable an informed decision as to the most appropriate antifungal treatment.

Keywords *Cryptococcus neoformans, Cryptococcus gattii*, molecular types, antifungal susceptibility

Introduction

Cryptococcosis is a systemic mycosis that affects humans and a large number of mammals, which presents most commonly as life-threatening meningoencephalitis [1]. Currently the two etiologic agents of disease form a species complex, i.e., *Cryptococcus neoformans* and *C. gattii*. Molecular epidemiological studies have identified eight major molecular types within this species complex. The molecular types of *C. neoformans* correlate with the serotypes as follows; VNI (=AFLP1) and VNII (=AFLP1A) correspond to serotype A, VNIII (=AFLP3) relates to the hybrid serotype AD, and VNIV (=AFLP2) corresponds to serotype D. In *C. gattii* such a correlation has not been observed in that the molecular types VGI (=AFLP4), VGII (=AFLP6), VGIII (=AFLP5) and VGIV (=AFLP7) all correspond to both serotypes, B or C [2, 3]. These major molecular types differ in their epidemiological and ecological features, clinical presentations and therapeutic outcomes. Infections caused by *C. gattii* often have a worse prognosis than those caused by *C. neoformans* [4]. *C. neoformans* infections occur worldwide and are an important cause of morbidity and mortality in immunocompromised hosts, especially HIV/AIDS patients. On the other hand, *C. gattii* is primarily the etiologic agent of infections in immunocompetent hosts and was considered to be restricted to tropical regions until the recent ongoing outbreak caused by the molecular type VGII in the temperate climates of Southwest of Canada and Northwest of the USA. This indicated the ability of this species to adapt to new environments [5].

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Antifungal susceptibility studies have reported a wide range of results depending on the species, serotypes, or the methodology (CLSI microdilution or Etest) used [6–10]. *C. gattii* was observed to be less susceptible than *C. neoformans* to itraconazole, fluconazole, ravuconazole, voriconazole, ketoconazole [6,7], and amphotericin B [7]. In addition, the MICs for fluconazole and amphotericin B obtained by the CLSI or Etest methods were significantly lower for *C. neoformans* serotype D strains when compared to serotype A [8]. However, other studies have found no differences in the susceptibilities between the two species [9] and among their serotypes A, D, B and C relative to amphotericin B, fluconazole, 5-fluorocytosine and voriconazole [10]. The present study aimed to determine if these inconsistent results are random or if they correlate to molecular type differences by investigating 67 Brazilian isolates.

Materials and methods

Strains studied

Eighteen isolates of *C. neoformans* molecular type VNI, 11 *C. gattii* molecular type VGI and 38 *C. gattii* molecular type VGII recovered from the Pathogenic Fungal Culture Collection of the Oswaldo Cruz Foundation, Rio de Janeiro, Brazil, were included in the study. The majority were isolated from cerebrospinal fluid (CSF; 61/67), and six from environmental sources (two VNI, one VGI and three VGII). Clinical VNI isolates were obtained from HIV positive (10) and HIV negative (five) patients, while those of VGII were recovered from four HIV positive and 18 HIV negative patients. All clinical VGI isolates were obtained from HIV negative patients. For 14 isolates (one VNI and 12 VGII) the HIV status of the patients from which they were isolated was unknown.

Antifungal susceptibility testing

The broth microdilution method was used to determine the MIC values [11], with a final concentration of 0.5×10^3 to 2.5×10^3 cells/ml and RPMI 1640 medium buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic. Growth and sterility control wells were included for each isolate tested. The following antifungal drugs were obtained as assay powders; albaconazole (J. Uriach & Co, S.A., Barcelona, Spain), amphotericin B (E. R. Squibb & Sons, Barcelona, Spain), 5-fluorocytosine (Hoffmann-La Roche, Basel, Switzerland), fluconazole (Pfizer, Madrid, Spain), itraconazole and ketoconazole (Janssen Research Foundation, Beerse, Belgium), ravuconazole (Bristol-Myers Squibb Company, New Brunswick, NJ, USA), and voriconazole (Pfizer, Madrid, Spain). The MIC endpoints were read after 72 h of incubation at 35°C. The MIC of amphotericin B was defined as the lowest concentration that caused 100% inhibition of growth, and the MICs of all other antifungal drugs were described as the lowest concentrations that produced 50% reduction in growth as compared to controls. The *Candida parapsilosis* strain ATCC 22019 and the *Candida krusei* strain ATCC 6258 were included as controls each time that a set of isolates was tested.

Genotyping

The molecular types were determined by *URA5*-RFLP analysis after amplification of the *URA5* gene from high molecular weight DNA. Amplification of the *URA5* gene was performed in a final volume of 50 µl. Each reaction contained 50 ng of DNA, 1 × PCR buffer [160 mM $(NH^4)_2SO^4$, 670 mM Tris-HCI (pH8.8 at 25°C), 0.1% Tween-20 – Bioline], 0.2 mM each of dATP, dCTP, dGTP, and dTTP (Roche Diagnostics GmbH, Switzerland), 3 mM magnesium chloride, 1.5 U BioTaq DNA polymerase (Bioline, Australia), and 50 ng of each primer URA5 (5′ ATGTCCTCCCAAGCCCTCGACTCCG 3′) and SJ01 (5′ TTAAGACCTCTGAACACCGTACTC 3′). PCR was performed for 35 cycles at 94°C with a 2 min initial denaturation, 45 s denaturation at 94°C, 1 min annealing at 61°C, 2 min extension at 72°C, and final extension cycle for 10 min at 72°C. The obtained PCR products were then double digested with the endonucleases *Sau*96I (10 U/µl) and *Hha*I (20 U/µl) for 3 h, and the fragments were separated by 3% agarose gel electrophoresis at 100 V [2]. The obtained *URA5*-RFLP patterns were assigned visually by comparison with the patterns obtained from the reference strains of each of the following molecular types; WM 148 (serotype A, VNI), WM 626 (serotype A, VNII), WM 628 (serotype AD, VNIII), WM 629 (serotype D, VNIV), WM 179 (serotype B, VGI), WM 178 (serotype B, VGII), WM 161 (serotype B, VGIII) and WM 779 (serotype C, VGIV) [12].

Statistics

Statistical analysis was performed using the program SPSS version 11.0 software (LEAD technologies, USA), and a P-value < 0.05 was used to define significance. The Wilcoxon test or the Mann-Whitney test was used for comparisons among the molecular types.

Results

The geometric means (GM) MIC_{90} and MIC ranges of the antifungal drugs tested are shown in Table 1. The range of MICs for each drug was; $<$ 0.03–0.25 µg/ml for albaconazole, 0.25–2 µg/ml for amphotericin B, $<$ 0.03–0.5 µg/ml for itraconazole, 0.25 to $>$ 64 µg/ml for 5-fluorocytosine, 0.25 to $>$ 64 µg/ml for fluconazole, $<$ 0.03–0.25 µg/ml for

Table 1 Minimum inhibitory concentrations of nine antifungal agents tested against 67 VNI, VGI and VGII cryptococcal isolates.

Mol. types (no. tested)	MIC parameter[a]	MIC (μg/ml)							
		ABZ	AMB	ITZ	5-FC	FCZ	KTZ	RVZ	VCZ
VNI (18)	Range	<0.03–0.06	0.25–2	<0.03–0.25	1–16	1–16	<0.03–0.125	<0.03–0.03	<0.03–0.125
	GM	0.019	0.56	0.058	3.43	2.42	0.032	0.014	0.038
	MIC_{90}	0.03	1	0.125	8	4	0.125	0.03	0.06
VGI (11)	Range	<0.03–0.06	0.25–1	0.03–0.25	1–8	0.5–4	<0.03–0.25	<0.03–0.125	<0.03–0.125
	GM	0.021	0.47	0.12	2.13	1.55	0.056	0.038	0.037
	MIC_{90}	0.03	0.5	0.25	4	4	0.125	0.06	0.06
VGII (38)	Range	<0.03–0.25	0.25–2	0.03–0.5	0.25–>64	2–64	<0.03–0.25	<0.03–0.25	0.03–0.5
	GM	0.042	0.62	0.15	4.00	6.08	0.06	0.043	0.10
	MIC_{90}	0.06	1	0.5	16	16	0.25	0.125	0.25

GM, geometric mean MIC; MIC90, MICs at which 90% of isolates were inhibited; ABZ, albaconazole; AMB, amphotericine B; ITZ, itraconazole; 5-FC, 5-fluorocytosine ; FCZ, fluconazole; KTZ, ketoconazole; RVZ, ravuconazole; VCZ, voriconazole.

ketoconazole, <0.03–0.25 μg/ml for ravuconazole and <0.03–0.5 μg/ml for voriconazole.

Comparisons of the MICs for the molecular types by Mann-Whitney test revealed that the molecular type VNI was more susceptible than VGI to itraconazole and ravuconazole ($P = 0.047$ and $P = 0.001$, respectively), and more susceptible than VGII to itraconazole ($P = 0.001$), ravuconazole ($P < 0.001$), fluconazole ($P = 0.001$), albaconazole ($P < 0.001$), voriconazole ($P < 0.001$) and ketoconazole ($P = 0.011$). There were no significant differences between VNI and VGII MICs to amphotericin B and 5-fluorocytosine ($P = 0.570$). The comparison of the two molecular types of *C. gattii*, VGI and VGII revealed that VGII was less susceptible than VGI to fluconazole ($P < 0.001$), albaconazole ($P = 0.004$), voriconazole ($P < 0.001$) and 5-fluorocytosine ($P = 0.045$). The only drug for which no differences in susceptibilities was found among VGI/VGII, VGI/VNI and VGII/VNI was amphotericin B (P-value = 0.053, 0.279, 0.465, respectively).

The GM of the MICs of the VNI isolates obtained from the HIV positive patients were significantly higher to fluconazole ($P = 0.025$) than those recovered from HIV negative patients. There were no significant differences among the VGII isolates recovered from the two host groups. The only clinical VGII isolate that had *in vitro* resistance to fluconazole (MIC ≥ 64) was obtained from an HIV negative patient.

Discussion

Major differences in the antifungal susceptibilities were observed among Brazilian cryptococcal isolates of the molecular types VNI, VGI and VGII. The molecular type VGII was less susceptible than VGI to four of the eight drugs tested (fluconazole, albaconazole, voriconazole and 5-fluorocytosine) and was less susceptible than VNI to six of the eight drugs tested (fluconazole, albaconazole, voriconazole, 5-fluorocytosine, itraconazole and ravuconazole). The only drug for which no difference was found in the susceptibilities of the different molecular types was amphotericin B. This differs from the findings reported by Khan *et al*. [7], who observed that the *C. gattii* serotype B was less susceptible than the *C. neoformans* serotype A to amphotericin B. However, this study analyzed only environmental isolates from India and used a different method, the E-test, the results of which are not always compatible to the CLSI microdilution method [13].

Results of other studies are similar to those described in this report, indicating that VGII is less susceptible to azoles regardless of the geographic region from which the isolates were obtained. A recent Australian study showed higher *in vitro* MIC's for the molecular type VGII than those found with VGI and the *C. neoformans* molecular types to voriconazole and fluconazole [14]. In addition, their analysis of the susceptibility to fluconazole showed that higher concentrations of drugs were needed to inhibit the growth of VGII isolates from the North and the West of Australia than the Vancouver Island (BC, Canada) outbreak VGIIa and VGIIb isolates [14]. Hagen *et al.*, investigating 350 *C. gattii* isolates from several countries, obtained similar results [15]. In their study the MIC values also varied according to the molecular types but did not vary with the geographical origin of the isolates. The VGII (= AFLP6) isolates were less susceptible than those of VGI (= AFLP4). Iqbal *et al.* also observed antifungal susceptibility differences related to genotypes [16]. Overall VGII isolates had higher *in vitro* MICs than VGI and VGIII. The VGIIc subgenotype was less susceptible than VGIIa, the most virulent subgenotype from the ongoing Vancouver Island (Canada) cryptococcosis outbreak. The combined results of all of the studies suggest that *C. gattii* molecular type VGII isolates were the least susceptible within the *C. neofomans/C. gattii* species complex, followed by isolates of the molecular types VGI, VNI and VNIV. In addition, differences in susceptibility were not limited to the major genotypes, but could also be found among subgenotypes. Molecular types

of *C. neoformans* and *C. gattii* are not equally distributed in the world, with VNIV being more often found in Europe, VGII the most common molecular type of *C. gattii* recovered in the America's, and VGI prevailing as the primary type of *C. gattii* in Oceania, Asia and Europe [18]. Therefore, the differences in susceptibilities may have important implications in the choice of treatment, representing a new factor influencing clinical outcomes/treatment response, especially in regions where there is a high genetic diversity of the *C. gattii* VGII population.

The results obtained to date indicate that the contradictory results observed in previous studies of antifungal susceptibilities among isolates of the *C. neoformans*/*C. gattii* species complex [6–10] may be attributed to the fact that the genotypes (molecular types) of the isolates were not taken into account or the use of different methodologies (CLSI microdilution or Etest) in the investigations.

It is unknown what causes the differences in the *in vitro* antifungal susceptibilities among isolates of different molecular types of the *C. neoformans/C. gattii* species complex. Sionov *et al*. [17] suggested that fluconazole resistance maybe due to chromosome duplication during prolonged azole therapy, a process that could be favoured in certain molecular types. Therefore, it seems further investigation of the genetic basis of these differences in antifungal susceptibilities of members of the *Cryptococcus neoformans/C. gattii* specie complex are warranted.

Fluconazole is the first choice for maintenance and prophylactic therapy of cryptococcosis [19]. In this study, isolates of *C. neoformans* recovered from HIV positive patients were less susceptible to fluconazole than those obtained from immunocompetent patients. In HIV positive individuals, maintenance of treatment last longer [19], relapses are more common [20] and previous treatment of oropharyngeal candidiasis with this antifungal may have occurred [21]. In addition, *C. neoformans* isolates express a high rate of innate heteroresistance to fluconazole [13], when combined with the longer exposure to fluconazole provide an opportunity for the emergence of resistance. This has been corroborated by Chowdhary *et al*. [22] who observed lower *in vitro* susceptibility of environmental isolates to fluconazole than those recovered from clinical samples in India. Furthermore, they observed increased MICs to fluconazole in serial isolates obtained after 1.5–2.5 months of therapy. This fact may partially explain why these authors observed lower susceptibility of VGI isolates to fluconazole when compared to VNI isolates, while in the present work VGI was only less susceptible than VNI to itraconazole and ravuconazole.

So far, the clinical implications of *in vitro* susceptibility differences are unknown, demanding further studies for a better understanding of the impact of the genotypes/molecular types on antifungal treatment and clinical outcome. Nevertheless, the reported data herein show a strong correlation between antifungal susceptibility and fungal genotype of members of the *Cryptococcus neoformans/C. gattii* complex. These findings indicate clearly that the determination of the molecular type of the causative agent in specific cases of cryptococcosis may provide important information that will allow for better guidance in the decision making for the best treatment choice, which subsequently would lead to a better disease outcome.

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