Antifungal susceptibility of Cryptococcus neoformans and Cryptococcus gattii isolates from decayed wood of trunk hollows of Ficus religiosa and Syzygium cumini trees in north-western India

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Background: We present antifungal susceptibility data on environmental isolates of Cryptococcus neoformans (serotype A, n = 117) and Cryptococcus gattii (serotype B, n = 65) cultured from decayed wood of trunk hollows of Ficus religiosa and Syzygium cumini trees.

Methods: Susceptibilities to amphotericin B, fluconazole, ketoconazole, itraconazole and voriconazole were determined by using Etest. The MICs were read after 48 h as per the guidelines provided by the manufacturer.

Results: The MIC90s and susceptibility ranges for C. neoformans isolates were as follows: 0.094 (0.004–0.25) mg/L for amphotericin B, 0.094 (0.004–0.75) mg/L for fluconazole, 0.064 (0.002–0.19) mg/L for ketoconazole, 0.064 (0.002–0.19) mg/L for itraconazole, 0.064 (0.002–0.19) mg/L for voriconazole, whereas for C. gattii isolates these were 0.125 (0.023–0.5) mg/L for amphotericin B, 0.75 (0.006–2) mg/L for fluconazole, 0.125 (0.003–0.19) mg/L for ketoconazole, 0.094 (0.004–0.125) mg/L for voriconazole. A comparison of the geometric means of MICs (mg/L) revealed that C. gattii was less susceptible than C. neoformans to amphotericin B (0.075 versus 0.051, P < 0.0003), fluconazole (2.912 versus 2.316, P < 0.003), itraconazole (0.198 versus 0.0344, P < 0.0001), ketoconazole (0.072 versus 0.037, P < 0.0001), and voriconazole (0.094 versus 0.023, P < 0.0001).

Conclusions: The antifungal susceptibility data obtained in this study indicate that the occurrence of primary resistance among environmental isolates of C. neoformans serotype A and C. gattii serotype B is rare, and serotype B isolates are less susceptible than serotype A isolates.

Keywords: Etest, fluconazole, itraconazole, voriconazole, amphotericin B

Introduction

Cryptococcosis is one of the leading pulmonary and meningeal mycoses of worldwide occurrence. The disease predominantly occurs in immunocompromised patients with underlying predisposing factors, such as organ transplantation, haematological malignancies, and advanced human immunodeficiency virus disease. The causative species include Cryptococcus neoformans (serotypes A, D, AD) and Cryptococcus gattii (serotypes B and C). Epidemiologically, these species differ from each other mainly with respect to geographic distribution, preference for natural habitat, pathobiology and host infectivity (immunocompetent versus immunocompromised) and genetics.1,2 Therapeutic management of cryptococcal infections usually consists of amphotericin B therapy with or without 5-flucytosine, whereas fluconazole is the drug of choice for prophylaxis and maintenance therapy. Owing to frequent instances of disease relapse, there is growing concern among clinicians about emergence of...
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antifungal resistance in C. neoformans and C. gattii during therapy or prophylaxis. Most reports of resistance have emerged in the setting of cryptococcal meningitis in AIDS patients after prolonged prophylaxis with fluconazole. This problem could have more serious dimensions in developing countries of Southeast Asia and Africa where large numbers of HIV-infected patients exist, and resources to treat the disease are inadequate or patients might receive suboptimal doses of fluconazole. In this context, recent reports of increased resistance to fluconazole in clinical isolates of C. neoformans originating from Cambodian and Indian patients are noteworthy.

Studies on antifungal susceptibilities of environmental isolates of C. neoformans or C. gattii are scanty and are based on a small number of isolates. No such information is available from Asia or Africa where cryptococcosis is a significant problem in HIV/AIDS patients. In this communication, we present data for a large number of environmental isolates of C. neoformans and C. gattii cultured from decayed wood inside trunk hollows of living trees of Ficus religiosa and Syzygium cumini in north-western India.

Materials and methods

Collection and identification of isolates

One hundred and eighty-two environmental isolates of C. neoformans serotype A (n = 117) and C. gattii serotype B (n = 65) from decayed wood inside trunk hollows of S. cumini living trees in Delhi/New Delhi metropolitan area, Amritsar city (Panjab), Meerut Cantt (Uttar Pradesh) and from F. religiosa trees in New Delhi cultured over a 6 year period were included for antifungal susceptibility testing. Identification of the C. neoformans and C. gattii isolates was initially done by the characteristic brown pigment developing in their yeast-like colonies on simplified niger seed medium with seed concentration increased to 70 g/L, and by verification of physiological characteristics employing the Vitek 2 Yeast ID system (bioMerieux, Marcy-l’Étoile, France). Confirmation of C. gattii isolates was done by their ability to grow on canavanine-glycine-bromothymol medium which was marked by change in colour of the medium from greenish yellow to blue. In case of any doubt, identity was also confirmed by D-proline assimilation.

Serotyping of the isolates was done by Crypto Check kit (Iatron Laboratories Inc., Tokyo, Japan).

Etest

The in vitro activity of the antifungal agents was determined by the Etest (AB Biodisk, Solna, Sweden) in accordance with the manufacturer’s instructions. The Etest was performed by inoculating 150 mm Petri dishes containing 60 mL of RPMI-1640 agar supplemented with 2% glucose and buffered to pH 7.0 with MOPS as recommended by the CLSI (formerly NCCLS). The inoculum was applied with cotton swabs using growth suspension prepared in 0.85% NaCl with a turbidity equivalent to that of a 0.5 McFarland standard. Plates were incubated at 35°C and read after 48–72 h. Reference strains Candida albicans ATCC 90 028 and Candida parapsilosis ATCC 22 019 were used for quality control. In the absence of CLSI susceptibility breakpoints for Cryptococcus species, only data on MICs for the isolates are presented. The isolates were tested in a blinded manner without knowing their species or the serotypes.

Statistical analysis

Scattergrams of the MICs for serotype A and serotype B isolates were compared by the Mann–Whitney test. Statistical analyses were performed with GraphPad Prism version 3.00 (San Diego, CA, USA). Statistical significance was defined as a P value <0.05.

Results

In vitro susceptibility

The comparative data on MIC against amphotericin B, fluconazole, itraconazole, ketoconazole and voriconazole as determined by Etest for C. gattii and C. neoformans isolates are presented in Figure 1. The MIC90s and susceptibility ranges for C. neoformans isolates (n = 117) were as follows: 0.094 (0.004–0.25) mg/L for amphotericin B, 4 (0.032–12) mg/L for fluconazole, 0.094 (0.004–0.75) mg/L for itraconazole, 0.064 (0.002–0.19) mg/L for ketoconazole, and 0.047 (0.006–0.125) mg/L for voriconazole, whereas for C. gattii isolates (n = 65) these were 0.125 (0.023–0.5) mg/L for amphotericin B, 8 (0.032–16) mg/L for fluconazole, 0.75 (0.006–2) mg/L for itraconazole, 0.125 (0.003–0.19) mg/L for ketoconazole, and 0.094 (0.004–0.125) mg/L for voriconazole.

Relationship with serotypes

A comparison of the geometric means (GMs) of MICs (mg/L) revealed that C. gattii serotype B showed significantly reduced susceptibility compared with C. neoformans serotype A to amphotericin B (0.075 versus 0.051, P = 0.0003), fluconazole (2.912 versus 2.316, P = 0.003), itraconazole (0.198 versus 0.0344, P < 0.0001), ketoconazole (0.072 versus 0.037, P < 0.0001), and voriconazole (0.045 versus 0.023, P < 0.0001) (Figure 1).

Discussion

There is paucity of reports on in vitro antifungal susceptibilities of environmental isolates of C. neoformans and C. gattii and these are based on small sample size. While most of the studies included isolates obtained from pigeon or bird droppings, the others have not indicated the source of origin. Our study is noteworthy in that it presents antifungal susceptibility data on the largest number of environmental isolates of C. neoformans and C. gattii reported so far, and all of them came from tree trunk hollows. In all probability, these isolates had no previous exposure to any of the antifungal agents tested in this study and hence the MIC values we have presented should be intrinsic. A comparison of the susceptibility profiles of environmental isolates published during 1996–2005 revealed that MIC90 values of antifungal agents have not changed noticeably. These observations are reinforced by our results. No noticeable differences were observed in the MICs for our environmental isolates obtained between 2000–02 and 2003–05, which are also in conformity with the global trends in antifungal susceptibility of clinical isolates of C. neoformans.

As reported in previous studies on environmental and clinical isolates, voriconazole exhibited highest activity against our environmental isolates of C. gattii as well as C. neoformans.
No CLSI susceptibility breakpoints for *Cryptococcus* species are currently available for any of the antifungal agents. Moreover, relationship of *in vitro* susceptibility results with clinical outcome is not yet clearly understood. Several technical problems pertaining to *in vitro* antifungal susceptibility of *Cryptococcus* species on RPMI medium used in CLSI methodology warrant attention. For instance, RPMI medium poorly identifies strains putatively resistant to amphotericin B and, therefore, to overcome this problem the use of yeast nitrogen base (YNB) medium or antibiotic 3 medium has been proposed. However, the MICs obtained on YNB medium in a recent comparative study did not predict the early clinical outcome better than the MICs obtained with RPMI medium. Etest on RPMI medium has been shown to yield higher MICs of fluconazole and lower MICs of amphotericin B than the CLSI method of broth dilution.

It is well known that *C. neoformans* (serotypes A, D and AD) and *C. gattii* (serotypes B and C) differ from each other in several characteristics, including epidemiology, pathogenicity, and clinical manifestations. Besides, minor to significant differences in the susceptibilities of the two species have been reported. Chen et al. reported that *C. gattii* isolates were less susceptible than *C. neoformans* to amphotericin B (*P* < 0.001). Likewise, a difference in susceptibilities to azoles (one dilution difference) has been reported. Using CLSI microdilution method, Trilles et al. found that while *C. gattii* was less...
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Susceptible to azoles than C. neoformans, this difference was not so conspicuous with respect to amphotericin B. This is contrary to the study of Chen et al.,36 who demonstrated that C. gattii is significantly less susceptible than C. neoformans to amphotericin B. A comparison of GMs of MICs in our study, however, revealed that C. gattii was less susceptible than C. neoformans to amphotericin B (P = 0.0003), fluconazole (P = 0.003), itraconazole (P < 0.0001), ketoconazole (P < 0.0001) and voriconazole (P < 0.0001). Recently, Dannaoui et al.34 reported susceptibility differences between serotypes A and D; serotype A was significantly less susceptible to amphotericin B (P < 0.03) and fluconazole (P = 0.0003) both by the CLSI method and Etest.

The reasons for the reduced susceptibility of C. gattii isolates to the drugs that inhibit or bind ergosterol in the cell membrane remain unclear. One of the possible mechanisms could be related to the relative differences in their ability to synthesize melanin. This is suggested by reports that pigmented C. neoformans cells were much more resistant to killing by amphotericin B and slightly so to fluconazole, possibly by a mechanism in which melanin present in the cell wall prevents the drug from reaching its active site.38,39 It may be pertinent to mention here that while studying the melanization properties of our environmental isolates on simplified niger seed agar and tobacco agar, we observed that C. gattii serotype B isolates produced more rapid and intense pigmentation than did C. neoformans serotype A isolates (Z. U. Khan and H. S. Randhawa, unpublished results). However, these differences in the antifungal susceptibility profiles between the two species or serotypes warrant further investigation.

In conclusion, the antifungal susceptibility data obtained in this study indicate that the occurrence of primary resistance against amphotericin B, fluconazole, itraconazole, ketoconazole and voriconazole among environmental isolates of C. neoformans serotype A and C. gattii serotype B is rare, and serotype B isolates are less susceptible than serotype A isolates.

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Transparency declarations

None to declare.

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