Published in final edited form as:

Mycoses. 2011 September ; 54(5): e438–e442. doi:10.1111/j.1439-0507.2010.01946.x.

Antifungal susceptibilities of *Cryptococcus neoformans* cerebrospinal fluid isolates from AIDS patients in Kenya

Rennatus Mdodo¹, Stephen A. Moser², Walter Jaoko³, John Baddley⁴, Peter Pappas⁴, Mirjam-Colette Kempf¹, Inmaculada Aban¹, Susan Odera³, and Pauline Jolly^{1,*}

¹University of Alabama at Birmingham (UAB), School of Public Health

²UAB Division of Laboratory Medicine, Department of Pathology

³University of Nairobi School of Medicine, Department of Medical Microbiology and Kenyatta National Hospital, Nairobi, Kenya

⁴UAB School of Medicine

Abstract

Background—Poor susceptibility of *Cryptococcus neoformans* to fluconazole (FLC) is a matter of concern among clinicians in Africa. The emergence of resistance to FLC was recently reported in Kenya but it is not known if it is widespread. Thus, there is need for more antifungal drug susceptibility studies in Kenya.

Objective—To measure the *in vitro* antifungal drug susceptibilities of incident *C. neoformans* isolates from acquired immunodeficiency syndrome patients in Kenya.

Methods—Antifungal susceptibility testing was performed on 67 *C. neoformans* isolates by broth microdilution method as outlined in the Clinical and Laboratory Standards Institute document M27-A3 using FLC, amphotericin B (AMB), voriconazole (VOR), ravuconazole (RAV), and flucytosine (5-FC). Isolates were grown on L-canavanine glycine bromothymol blue medium for serotype identification.

Results—Six percent of the isolates were identified as *C. neoformans* var. *gattii* serotype B or C and 94% as *C. neoformans* var. *neoformans*. All isolates tested were susceptible to AMB, VOR and RAV (100%), and high susceptibilities were seen to FLC (97%), and 5-FC (90%). Only 3% and 10% of the isolates' susceptibility to FLC and 5-FC respectively, was dose dependent or intermediate.

Conclusions—These results demonstrate high susceptibilities of incident *C. neoformans* isolates to FLC and AMB, antifungals used for treatment of cryptococcal meningitis in Kenya.

Keywords

Cryptococcus neoformans; susceptibility; fluconazole; amphotericin B; serotypes; Kenya

Introduction

Cryptococcus neoformans is an environmental saprophyte that commonly infects immunocompromised patients causing cryptococcal meningitis (CM) [1]. The three varieties

*Corresponding author, Department of Epidemiology, School of Public Health, University of Alabama at Birmingham, 1665 University Boulevard, RPHB 217, Birmingham AL 35294-0022. Tel: 205-934-1823 Fax: 205-975-3329 jollyp@uab.edu .

Competing interests: There are no conflicts of interests for all authors.

reported in Africa are *C. neoformans* var. *neoformans*, *C. neoformans* var. *grubii* and *C. neoformans* var. *gattii* [2,3]. *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii* have a worldwide distribution, while *C. neoformans* var. *gattii* is confined to tropical/subtropical regions, and in the Northeast United States and Vancouver area [4,5]. The incidence of *Cryptococcus* infections has increased dramatically over the years in many countries due to the HIV epidemic [6,7].

Studies in Africa have demonstrated high disease burden, morbidity and mortality associated with CM among patients with Acquired Immunodeficiency Syndrome (AIDS) [8-10]. Fluconazole (FLC) is the drug of choice for treatment of CM and its free access through the Diflucan Partnership Program has greatly improved the quality of life for many patients in Africa. However, concerns about the emergence of FLC resistant strains of *C. neoformans* have been raised by various investigators [3,6,11,13]. Reduced susceptibility of *C. neoformans* to FLC has been reported in a number of sub-Saharan countries including Kenya, Uganda, Rwanda and South Africa.² Studies in Kenya and South Africa showed FLC resistance at a frequency of 11.2% and 12.7% respectively [3,12]. It is speculated that reduced susceptibility to FLC in Kenya may be due to poor prescription practices and under dosages [3].

With wider use of FLC for treatment of CM as the HIV positive population increases, antifungal resistance is likely to be on the rise. Antifungal resistance will increase treatment costs in these resource poor countries where healthcare infrastructures are already strained [12]. We conducted this study because of the gravity of the potential problems posed by antifungal drug resistance and the need for more antifungal susceptibility data in Kenya. For the conduct of this study we measured the *in vitro* antifungal drug susceptibilities of incident *C. neoformans* isolates from AIDS patients at two referral hospitals in Kenya.

Materials and Methods

Isolates and clinical data

A total of 67 *Cryptococcus* isolates from the cerebrospinal fluid (CSF) of HIV positive patients from Kenyatta National Hospital and Mbagathi District Hospital in Nairobi Kenya were used for this study. CSF was cultured on Sabouraud's dextrose agar (BD, Sparks, MD, USA) to obtain the isolates. The presence of *C. neoformans* was confirmed by a positive urease test. The isolates were transferred into agar slants and stored at room temperature or aliquoted in a glycerin/milk solution and stored at -80 °C at the Kenyatta National Hospital microbiology laboratory. These isolates were then shipped to a laboratory at the University of Alabama at Birmingham (UAB) for antifungal susceptibility testing and confirmation of identification.

Demographic data including gender, age, baseline CD4 counts, education and in-hospital mortality were obtained using a structured questionnaire and patient medical records. All participants included in the study gave informed consent to have their CSF collected and their clinical records reviewed. Ethical approval to conduct this study was obtained from the UAB Institutional Review Board and the Kenyatta National Hospital Ethics and Research Committee. Data analysis was conducted using SAS, version 9.1 (SAS Institute Inc., Cary, North Carolina, USA).

Antifungal drugs

We tested six antifungal drugs from the manufacturers as follows: FLC and voriconazole (VOR) (Pfizer, Inc., N.Y, USA), Amphotericin B (AMB) (Sigma St. Louis, MO, USA) and ravuconazole (RAV) (Bristol-Myers Squibb, Princeton, N.J, USA), flucytosine (5-FC) (Sigma St. Louis, MO, USA). Some of the drugs tested are not available for use in Kenya,

but knowledge of their potency in comparison to AMB and FLC will be useful in guiding antifungal drug use in the future.

Broth microdilution susceptibility testing and biochemical characterization

Minimum inhibitory concentrations (MICs) to AMB, FLC, VOR, RAV, POS, and 5-FC were determined by broth microdilution per CLSI document M27-A3 [14]. Inocula were prepared from a 48 hr culture on sabouraud's dextrose agar (BD, Sparks, MD, USA). Interpretive criteria for FLC (susceptible ≤ 8 $\mu\text{g/ml}$, susceptible dose dependent, 16–32 $\mu\text{g/ml}$ and resistant, ≥ 64 $\mu\text{g/ml}$), 5-FC (susceptible ≤ 4 $\mu\text{g/ml}$, intermediate, 8–16 $\mu\text{g/ml}$ and resistant ≥ 32 $\mu\text{g/ml}$), AMB, (susceptible ≤ 1) were used [18]. Interpretive criterion for VOR and RAV (susceptible ≤ 1) is based on the study by Pfaller, *et al.* [11].

Drug concentrations tested were 0.03–16 $\mu\text{g/ml}$ (AMB), 0.125–64 $\mu\text{g/ml}$ (FLC), 0.015–8 $\mu\text{g/ml}$ (RAV and VOR) and 0.015–64 $\mu\text{g/ml}$ (5-FC). Final dilutions were made in RPMI 1640 medium (Sigma St. Louis, MO, USA) buffered to pH 7.0 with 0.165 M 3-(N-morpholino) propanesulfonic acid (MOPS) buffer (Sigma St. Louis, MO, USA). MIC values were determined, after incubation at 72 hours at 35°C, visually with endpoints based on the lowest concentration that produced a 50% inhibition in growth compared to that of the drug-free control for azoles and 5-FC and 100% inhibition for AMB. *Candida parapsilosis* (ATCC 22019) and *C. krusei* (ATCC 6258) were included with each testing for quality control. Isolates were grown on L-canavanine glycine bromothymol blue medium (CGB) to identify them to species level as described by Kwon-Chung *et al.* [15].

Results

Patient characteristics

Baseline characteristics of the patients are summarized in Table 1. Patient median age was 35 (range: 20–53) years. Males comprised the greatest proportion of the patients (65%). Approximately one-third (32%) of the patients were currently or previously on ART, and two-thirds (66%) had a history of antifungal drug use. Most patients had CD4 count <200 cells/ μL (94%), were hospitalized for the first time (90%), and died during hospitalization (60%).

Antifungal drug susceptibility testing

Six percent (4/67) of the isolates were identified as *C. neoformans* var. *gattii* (serotype B or C) and 94% (63/67) as *C. neoformans* var. *neoformans* (serotype A or D). *In vitro* susceptibilities of these isolates to six antifungal agents are summarized in Table 2. All isolates were susceptible to AMB (range 0.5–1.0 $\mu\text{g/ml}$). Ninety seven percent of the isolates were susceptible to FLC (range 0.25–16.0 $\mu\text{g/ml}$) and 90% to 5-FC (range 1.0–16.0 $\mu\text{g/ml}$). Only 3% and 10% of the isolates' susceptibility to FLC and 5-FC respectively, was dose dependent or intermediate. All isolates were susceptible to RAV (range 0.015–0.25 $\mu\text{g/ml}$), and VOR (0.015–0.25 $\mu\text{g/ml}$).

Discussion

This study highlights the existence of two *Cryptococcus* serotypes in our isolates; *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii*, indicating the possibility of more than one environmental source of the fungus in Kenya. *C. neoformans* var. *neoformans* is usually found in pigeon excreta while *C. neoformans* var. *gattii* is found commonly in the tropics where *eucalyptus* trees are plentiful [16]. Kenya provides an ideal environment for both species. In this study *C. neoformans* var. *neoformans* was more prevalent in our study population compared to *C. neoformans* var. *gattii*. Similar findings were reported in Kenya

recently [3]. It will be interesting to conduct a country wide environmental survey to map the distribution of *C. neoformans* serotypes to understand the clinical significance of *C. neoformans* var. *gattii*. This serotype is not very common in Kenya but its clinical significance may be increasing [3].

Reports on the emergence of *C. neoformans* with decreased susceptibility to FLC in Africa are of great concern as this is the drug widely used for treatment of CM. The introduction of free FLC in Kenya through the Diflucan Partnership Program has saved lives but has also seen a huge increase in its use [17]. This has prompted concerns that the drug is over prescribed and may lead to widespread resistance. A study by Bii *et al.* [3], confirmed these fears when it reported *in vitro* resistance to FLC as high as 11.5% [3]. In contrast, our findings show high susceptibility to FLC with only 3% of the tested isolates' susceptibility being dose dependent. High susceptibility was also seen to AMB and the other antifungals tested.

Our findings are surprising as we had hypothesized high antifungal drug resistance due to the widespread use of FLC in hospitals. It is difficult to predict why we found no resistance compared to results obtained by Bii *et al.* [3]. It is likely that the incident isolates used in our study were tested before they developed storage induced resistance. The difference may also be explained by the methods used. Bii *et al.* [3] used the commercial frozen plate kit (Eiken Chemical Co. LTD, Tokyo). Although this method was evaluated to show over 90% agreement with the CLSI method that we used, it may explain the contrasting results obtained [19].

Clinical response to FLC occurs when the MIC is <16 µg/ml [1]. With efficacy as high as 97% FLC is still useful as the drug of choice for treatment of CM in Kenya. This is welcome news for Kenya where AMB is too expensive for most patients who must rely solely on FLC for treatment of CM. Although poor prescription practices and under-dosages may be a problem in Kenya we find no evidence of widespread resistance to AMB and FLC in this study. Safe guards to prevent the development of resistance to these life saving antifungals must be adopted as suggested by Bii *et al.* [3].

Conclusion

Our results show that antifungal resistance remains uncommon among *C. neoformans* isolates in Kenya. We also show that these drugs are extremely potent against the clinical isolates of *C. neoformans*. However, despite these positive findings measures to control how these drugs are used are necessary. The recommendations by Bii *et al.* [3] to control irrational use of antifungal drugs and to support the establishment of a nationwide antifungal drug resistance surveillance program in Kenya are warranted.

Acknowledgments

The authors would like to thank all the patients who participated in this study. We are grateful to the staff, interns and students of Kenyatta National Hospital, Mbagathi District Hospital, University of Nairobi Microbiology Department, Kenya AIDS Vaccine Initiative and UAB Division of Laboratory Medicine, Department of Pathology. We thank CDC researchers, Drs Tom Chiller, Ben Park and Beth Arthington-Skaggs, and University of Nairobi faculty Dr. Enoch Omonge for their contributions in this study.

Financial support: This study was supported by the Minority Health International Research Training (MHIRT) grant no. T37-MD001448 from the National Center on Minority Health and Health Disparities, National Institutes of Health, Bethesda, MD, USA, the Department of Medical Microbiology, University of Nairobi, and Kenyatta National Hospital, Nairobi, Kenya

References

1. Mitchell TG, Perfect JR. Cryptococcosis in the AIDS era-100 years after the discovery of *Cryptococcus neoformans*. Clin Microbiol Rev. 1995; 8:515–548. [PubMed: 8665468]
2. McCarthy K, Morgan J, Wannemuehler K, et al. Population-based surveillance for cryptococcosis in an antiretroviral-naïve South African province with high HIV seroprevalence. AIDS. 2006; 20:2199–2206. [PubMed: 17086060]
3. Bii C, Makimura K, Abe S, et al. Antifungal drug susceptibility of *Cryptococcus neoformans* from clinical sources in Nairobi, Kenya. Mycoses. 2007; 50(1):25–30. [PubMed: 17302744]
4. Franzot SP, Salkin IF, Casadevall A. *Cryptococcus neoformans* var. *grubii*: separate varietal status for *Cryptococcus neoformans* serotype A isolates. J Clin Microbiol. 1999; 37:838–840. [PubMed: 9986871]
5. Casadevall, A.; Perfect, JR., editors. *Cryptococcus neoformans*. American Society for Microbiology; Washington, D.C: 1998. Epidemiology; p. 351-380.
6. Brandt ME, Pfaller MA, Hajjeh RA, et al. Trends in antifungal drug susceptibility of *Cryptococcus neoformans* isolates in the United States: 1992 to 1994 and 1996 to 1998. Antimicrob Agents Chemother. 2001; 45:3065–3069. [PubMed: 11600357]
7. Park BJ, Wannemuehler KA, Marston BJ, Govender N, Pappas PG, Chiller TM. Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. AIDS. 2009; 23:525–530. [PubMed: 19182676]
8. Bicanic T, Harrison TS. Cryptococcal meningitis. Br Med Bull. 2005; 72:99–118. [PubMed: 15838017]
9. Okongo M, Morgan D, Mayanja B, Ross A, Whitworth J. Causes of death in a rural, population-based human immunodeficiency virus type 1 (HIV-1) natural history cohort in Uganda. Int J Epidemiol. 1998; 27:698–702. [PubMed: 9758128]
10. French N, Gray K, Watera C, et al. Cryptococcal infection in a cohort of HIV-1-infected Ugandan adults. AIDS. 2002; 16:1031–1038. [PubMed: 11953469]
11. Pfaller MA, Messer SA, Boyken L, et al. Global trends in the antifungal susceptibility of *Cryptococcus neoformans* (1990 to 2004). J Clin Microbiol. 2005; 43:2163–2167. [PubMed: 15872236]
12. Bicanic T, Harrison T, Niepieklo A, Dyakopu N, Meintjes G. Symptomatic Relapse of HIV-Associated Cryptococcal Meningitis after Initial Fluconazole Monotherapy: The Role of Fluconazole Resistance and Immune Reconstitution. Clin Infect Dis. 2006; 43:1069–1073. [PubMed: 16983622]
13. Sar B, Monchy D, Vann M, Keo C, Sarthou JL, Buisson Y. Increasing in vitro resistance to fluconazole in *Cryptococcus neoformans* Cambodian isolates: April 2000 to March 2002. J Antimicrob Chemother. 2004; 54:563–565. [PubMed: 15254027]
14. Clinical and Laboratory Standards Institute. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts: Approved Standard. 3rd edn. Clinical and Laboratory Standards Institute; Wayne, PA: 2007. Document M27-A3
15. Kwon-Chung KJ, Polacheck I, Bennett JE. Improved diagnostic medium for separation of *Cryptococcus neoformans* var. *neoformans* (serotypes A and D) and *Cryptococcus neoformans* var. *gattii* (serotypes B and C). J Clin Microbiol. 1982; 15:535–537. [PubMed: 7042750]
16. Kwon-Chung KJ, Bennett JE. High prevalence of *Cryptococcus neoformans* var. *gattii* in tropical and subtropical regions. Zentralbl Bakteriol Mikrobiol Hyg [A]. 1984; 257:213–8.
17. Diflucan Partnership Program. [Retrieved on 01/25/10]. from: <http://www.diflucanpartnership.org/en/welcome/>
18. Nguyen MH, Yu CY. In vitro comparative efficacy of voriconazole and itraconazole against fluconazole-susceptible and -resistant *Cryptococcus neoformans* isolates. Antimicrob Agents Chemother. 1998; 42:471–472. [PubMed: 9527812]
19. Makimura K, Oguri T, Mikami Y, et al. Multicenter evaluation of commercial frozen plates for microdilution both antifungal susceptibility testing of yeasts and comparison of MIC limits recommended in NCCLS M27-A2. Microbiol Immunol. 2005; 49:97–106. [PubMed: 15722594]

Table 1

Baseline characteristics of the study patients admitted at KNH and MDH between August 2008 and March 2009.

Variable	Total N = 65 (%)
Age, median years (range)	35 (20-53)
Male sex	42 (65)
Secondary education	34 (52)
Employment	46 (71)
ART, current or previous	21 (32)
Previous antifungal treatment	43 (66)
CD4 ⁺ T lymphocyte count <200 (cells /mm ³)	47/50 (94)*
In-hospital mortality	38/62 (61)*
First time hospitalization	56/62 (90)*

* Variables with denominators are listed to show missing data.

In vitro antifungal drug susceptibilities of incident *C. neoformans* isolates from AIDS patients in Kenya.

Table 2

Antifungal drug concentration (µg/ML)	FLC [N=66] n (%)	AMB [N=67] n (%)	5-FC [N=67] n (%)	RAV [N=59] n (%)	VOR [N=67] n (%)
0.015				33 (56)	4 (6)
0.03				7 (12)	14 (21)
0.06				17 (29)	28 (42)
0.125				1 (1.5)	17 (25)
0.25	2 (3)			1 (1.5)	4 (6)
0.5	1 (2)	15 (22)			
1	5 (8)	52 (78)	3 (4.6)		
2	16 (24)		23 (34.2)		
4	32 (48)		35 (52)		
8	8 (12)		3 (4.6)		
16	2 (3)		3 (4.6)		
Range (µg/ML)	0.25-16	0.5-1	1-16	0.015-0.25	0.015-0.25
MIC ₅₀	4	1	2	0.015	0.06
MIC ₉₀	8	1	4	0.25	0.25

MIC = Minimum Inhibitory Concentration

MIC₅₀ and MIC₉₀ = MICs at which 50% and 90% of the isolates were inhibited, respectively.