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SUSCEPTIBILITY OF *CRYPTOCOCCUS NEOFORMANS* AND *CRYPTOCOCCUS GATTII* FROM CLINICAL AND ENVIRONMENT SOURCES IN NAIROBI, KENYA
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SUSCEPTIBILITY OF *CRYPTOCOCCUS NEOFORMANS* AND CRYPTOCOCCUS GATTII FROM CLINICAL AND ENVIRONMENT SOURCES IN NAIROBI, KENYA

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ABSTRACT

***Objective*: To determine anti-fungal susceptibility of *Cryptococcus neoformans* and *Cryptococcus gattii* from environmental and clinical sources in Nairobi, Kenya.**
***Design*: Prospective study.**
***Setting*: Kenya Medical Research Institute, Mycology laboratory, Nairobi, Kenya.**
***Subjects*: A total of 123 isolates were tested for their susceptibility to fluconazole (FLC), amphotericin B(AMP) and fluorocytosine (5FC). Clinical isolates were 70(66 *Cryptococcus neoformans* and 4 *Cryptococcus gattii*) while environmental isolates were 53(41 *C. neoformans* and 12 *C. gattii*). The isolates were characterised using various phenotypic tests including microscopic morphology, physiological and biochemical tests (API 20 Caux), pigmentation on bird seed agar and reaction on canavanine-glycine-bromthymolblue agar. European Committee on Anti-microbial Susceptibility Standards (EUCAST) was used as the reference method for susceptibility testing.**
***Results*: Most *C. neoformans* isolates; clinical (61/66; 92.4%) and environmental (38/41; 92.7%) were susceptible to FLC. The number of *C. neoformans* isolates inhibited at susceptible dose dependent (SDD) range (16-32μg/ml) by FLC were clinical (4/66; 6.1%) and environmental (2/41; 4.9%). One *C. neoformans* isolate each; clinical (1/66; 1.5%) and environmental (1/41; 2.4%) was resistant to FLC. All *C. gatti* isolates from clinical and environmental were fully susceptible to FLC. The percentage of *C. neoformans* isolates that were susceptible (S) (MIC ≤ 1.0 μg/ml) to AMP were; clinical(52/66; 90.2%) and environmental (37/41; 78.8%) while the rest were susceptible dose dependent (SDD) with MIC (2-8μg/ml). Reduced susceptibilities to 5FC was displayed in all clinical and environmental *C. neoformans* and *C. gatii* isolates; for instance resistance to 5FC was reported in *C. neoformans*; clinical (8/66; 12.1%) and environmental (1/41; 2.4 %). Among the *C. gattii* isolates there was also decreased susceptibility to 5FC with Minimum Inhibition Concentration (MIC) range of between 0.5-32 μg/ml. There were no significant differences in susceptibility ranges among all the clinical and environmental isolates.**
***Conclusion*:This study demonstrated reduced susceptibilities among *C. neoformans* and *C. gattii* isolates to commonly used anti-fungal drugs.**

INTRODUCTION

Anti-fungal susceptibility testing results of clinically significant fungal strains are of interest to physicians, enabling them to adopt appropriate strategies for empiric and prophylactic therapies (1). The need for reproducible, clinically relevant anti-fungal susceptibility. Testing has been prompted by the increasing number of invasive fungal infections, the expanding use of new and established anti-fungal agents, and recognition of anti-fungal resistance as an important clinical problem (1, 2).

Cryptococcus neoformans and *C. gattii* are important fungal pathogens that cause predominantly fatal mycotic infections in immunocompromised patients (3,4). *C. neoformans* has historically been divided into three varieties of five serotypes based on antigenicity of the capsule: *C. neoformans* var. *grubii* (serotype A), *C. neoformans* var. *gattii* (serotypes B and C), *C. neoformans* var. *neoformans*(serotype D), and one hybrid (serotype AD) (5). In 2002, *C. neoformans* var. *gattii* (serotypes B and C) was awarded species status and renamed *Cryptococcus gattii* (6). Currently the two species are referred to as *Cryptococcus neoformans-Cryptococcus gattii* species complex.The different types of *C. neoformans* are found worldwide in environmental niches such as soil, bird excreta, or in the case of *C. gattii* on surfaces of *Eucalyptus* or other tropical trees (7,8).

Mortality due to meningitis caused by *C. neoformans-C.gattii* species complex in HIV-infected patients in Kenya and other sub-Sahara countries is high (3, 9). However, limited data exist on the occurrence and anti-fungal susceptibilities of this pathogenic yeast. In sub-Saharan Africa, cryptococcal meningitis occurs in 30% of AIDS patients and is likely to remain a substantial cause of death in these patients unless highly active antiretroviral therapy becomes available (10, 11). Until such a time, treatment with anti-fungal agents, including long-term, suppressive anti-fungal regimens, remains the only recourse. The widespread use of FLC maintenance therapy in HIV is a risk factor for the emergence of isolates with reduced susceptibility. Natural selective pressures exerted on micro-organisms by routine, inappropriate, irrational or excessive use of anti-microbial drugs are risk factors for the development of anti-microbial resistance(2). Anti-fungal resistance in tropical developing countries is more likely due to; unrestricted availability of anti-microbial drugs, poor prescription practices, suboptimal therapeutic regimens, blindempiric prescribing practices that are not epidemiologically directed, and lack of laboratory capacity or skilled personnelfor susceptibility testing is a receipt for spread of anti-microbial resistance(1,12). Amphotericin B with or without 5 FC remains the 'reference standard' anti-fungal drug for induction therapy (13). A high oral dose of FLC with 5FC is not as effective as AMP with 5FC (13). In Kenya, FLC is the most commonly administered drug for the treatment of cryptococcosis. The need for lifelong FLC maintenance therapy due to high relapse rates of cryptococcosis in HIV / AIDS raises concerns over anti-fungal resistance in developing countries(14). Development of resistance to FLC would be devastating for the management of this fatal disease. It is therefore important for public health agencies to monitor for changes in FLC susceptibility. Due to the environmental source of *C. neoformans* and *C. gatti*i species complex (7,15) comparison of susceptibility profiles of both environmental and clinical isolates is essential for monitoring of resistance. This study is the first to document the anti-fungal susceptibility profiles of both clinical and environmental *C.neoformans* and *C. gattii* isolates in Kenya.

MATERIALS AND METHODS

A total of 123 isolates of *C. neoformans* and *C. gattii* isolates were subjected to susceptibility to FLC, AMP and 5FC. Clinical isolates were 70 (66 C. neoformans and 4 C. gattii) whereas environmental isolates were 53 (41 *C. neoformans* and 12 *C. gattii*). The isolates were confirmed using various phenotypic tests including microscopic morphology, physiological and biochemical tests (API 20 Caux, Biomerieux, Marcy l'Etoile, France), pigmentation on bird seed agar and reaction on canavanine-glycine-bromthymol blue agar.

Anti-fungal susceptibility testing was performed in accordance to the reference method for broth dilution anti-fungal susceptibility testing of yeast, European Committee for Anti-microbial Susceptibility TestingDefinitive Revision (EDef 7.1 and EDef 7.2) (16,17). *Candida parapsilosis* (ATCC 22019) and *Candida krusei* (ATCC 6258) were incorporated as quality control strains in each set of experiments (17).

Standard powders of AMP (Sigma–Aldrich, Munich, Germany), 5FC (Sigma–Aldrich) and FLC (Pfizer, Karlsruhe, Germany), were used. Amphotericin B was dissolved in DMSO at the proposed stock solution of 2 mg/ml. Fluconazole was dissolved in methanol at the proposed stock solution of 125 mg / ml. Fluorocytosine was dissolved in sterile distilled water at initial stock solution of 10 mg / ml. The preparation of the different working solutions was performed as described in EDef 7.2 document (17). Exactly 100 µL from each of the tubes containing the corresponding concentration (2 x final concentrations) of anti-fungal agent was dispensed into sterile plastic, disposable, 96 well microdilution plates. The final concentrations were in the range of 0.125-64µg / ml for 5FC and FLC and 0.03-16µg / mlfor AMP.

The MICs of AMP, 5FC and FLC, were determined according to the reference procedures of the EDef 7.2 document (17). Testing was performed with RPMI 1640 medium supplemented with 0.2% glucose in flat-bottomed micro dilution plates. The pH of the test medium was seven. An inoculum size of 105 cfu / mL was used. The MIC endpoints were determined spectrophotometrically after 48 hours and 72 hours of incubation at 30ºC. The endpoint of AMP MIC was defined as the lowest drug concentration

that resulted in a reduction in growth by 90% or more, compared with that of a drug-free growth control well. The MIC endpoint for 5FC and FLC was defined as a 50% reduction in optical density. The interpretive breakpoints proposed by th eEDef 7.2 document were used (17). Isolates were classified according to their MIC as susceptible (S), susceptible dose-dependent (S-DD), and resistant (R)(17). The MIC^{50} and MIC^{90} values were determined asconcentrations where 50% or 90%, respectively, of all fungal isolates were inhibited bythe test anti-fungal drug. Statistical analysis to compare susceptibility profiles of clinical and environmental isolates was done using Epi Info 2000 software (version 1.1.1), chi square at 95% ($p \leq 0.05$) confidence limits. This was also used to compare the susceptibilities of the two *Cryptococcus* species.

RESULTS

The number and percentage of isolates inhibited at each concentration of FLC over the full dilution series is summarised in Table 1.The MIC^{50} and MIC^{90} of both clinical and environmental *C. neoformans* to fluconazole were4 μg/ml and 8 μg/ml respectively. The MIC^{50} and MIC^{90} of both clinical and environmental *C. gattii* to fluconazole were 8 μg/ml and 8 μg/ml respectively. The clinical and environmental *C. neoformans* isolates did not differ significantly in their susceptibility profiles to fluconazole; Susceptible(S)≤ 8μg/ml, Susceptible dose dependent (S-DD) 16-32 μg/ml, and Resistance (R) ≥ 64 μg/ml(P>0.05). All the *C. gattii* isolates were fully susceptible to FLC.

Table 1
In-vitro susceptibility of environmental and clinical Cryptococcus neoformans and Cryptococcus gattii to fluconazole (FLC)

		Number (%) of isolates inhibited at each category	
Isolate source	Category(MIC Range (μg/Ml)	*Cryptococcus neoformans*	*Cryptococcus gattii*
Environmental	Susceptible (S) ≤ 8	38/41 (92.7)	12/12 (100%)
Clinical	Susceptible (S) ≤ 8	61/66 (92.4)	4/4 (100%)
		(P=0.961)	
Environmental	Susceptible dose dependent (S-DD) (16-32)	2/41 (4.9)	n/a
Clinical	Susceptible dose dependent (S-DD) (16-32)	4/66 (6.1)	n/a
		(P=0.797)	
Environmental	Resistant (R) ≥ 64	1/41 (2.4)	n/a
Clinical	Resistant (R) ≥ 64	1/66 (1.5)	n/a
		(P=0.733)	

(N/A is not applicable).

The number and percentage of isolates inhibited at each concentration of AMP over the full dilution series is summarised in Table 2. The MIC^{50} and MIC^{90} of clinical *Cryptococcus neoformans* to amphotericin B were 2 μg/ml and 4 μg/ml respectively whereas MIC^{50} and MIC^{90} of amphotericin B to environmental *Cryptococcus neoformans* were 0.5 μg/ml and 2 μg/ml respectively. On the other hand MIC^{50} and MIC^{90} of AMP to clinical *Cryptococcus gattii* was 1.0 μg/ml and 4μg/ml respectively while MIC 50 and MIC 90 of AMP to environmental *Cryptococcus gattii* was 0.5 μg/ml and 1 μg/ml respectively. The clinical and environmental *C. neoformans* isolates did not differ significantly in their susceptibility profiles to AMP; Susceptible(S) ≤ 1 μg/ml (P>0.05); neither was there significant differences in susceptibility among clinical and environmental *C. gattii* isolates, (P>0.05).

Table 2
In-vitro susceptibility of environmental and clinical Cryptococcus neoformans and Cryptococcus gattii to amphotericin B (AMP)

		Number (%) of isolates inhibited at each category	
Isolate source	Category(MIC Range (μg/Ml)	*Cryptococcus neoformans*	*Cryptococcus gattii*
Environmental	≤1.0	37/41 (90.2)	11/12(91.7)
Clinical	≤1.0	52/66 (78.8)	3/4 (75)
		(P=0.125)	(P=0.398)
Environmental	2.0-8.0	4/41 (9.8)	1/12 (8.3)
Clinical	2.0-8.0	14/66 (21.2)	1/4 (25)
		(P=0.125)	(P=0.398)
Environmental	≥ 16	n/a	n/a
Clinical	≥ 16	n/a	n/a

The number and percentage of isolates inhibited at each concentration of 5FC over the full dilution series is summarised in Table 3. The MIC^{50} and MIC^{90} of five fluorocytosine to clinical *Cryptococcus neoformans* were 16 μg/ml and 64 μg/ml respectively whereas the MIC50 and MIC^{90} of five fluorocytosine to environmental Cryptococcus neoformanswere 8 μg/ml and 32 μg/ml. On the other hand MIC^{50} and MIC^{90} of five fluorocytosine to both clinical and environmental *Cryptococcus gattii* were 4 μg/ml and 16 μg/ml respectively. The clinical and environmental *C. neoformans* isolates did not differ significantly in their susceptibility profiles to 5FC; Susceptible(S) ≤ 4 μg/ml , Susceptible dose dependent (S-DD) 8-32 μg/ml and Resistance (R) ≥ 64 μg/ml (P>0.05). There was no significant difference in susceptibility profiles of clinical and environmental *C. gattii* Susceptible (S) (P = 0.78), susceptible dose dependent (S-DD) (P>0.05).

Table 3
In-vitro susceptibility of environmental and clinical Cryptococcus neoformans and Cryptococcus gattii to Fluorocytosine (5FC)

		Number (%) of isolates inhibited at each category	
Isolate source	Category(MIC Range (μg/Ml)	*Cryptococcus neoformans*	*Cryptococcus gattii*
Environmental	≤ 4	11/41 (26.8)	7/12 (58.3)
Clinical	≤ 4	12/66 (18.2)	2/4 (50)
		(P=0.292)	(P=0.78)
Environmental	8.0-32.0	29/41 (70.8)	5/12 (41.7)
Clinical	8.0-32.0	46/66 (69.7)	1/4 (75)
		(P= 0.910)	(P=0.56)
Environmental	≥ 64	1/41 (2.4)	n/a
Clinical	≥ 64	8/66 (12.1)	1/4 (25)
		(P=0.08)	n/a

N/A Not -applicable

DISCUSSION

In this study, majority of the isolates analysed were *Cryptococcus neoformans* (87%) and the rest were *Cryptococcus gattii*. Similar studies in Kenya and other parts of the world have also reported a higher frequency of *C. neoformans* as compared to *C. gattii* from both clinical and environmental sources (18,19). HIV/AIDS is the major predisposing factor to cryptococcal infections especiallyin sub-Saharan Africa which is the epicenter of AIDS pandemic (3,20). *Cryptococcus gattii* isolates are predominant in tropical and subtropical regions on surfaces of *Eucalyptus* or other trees (7, 21, 22). Widespread cultivation of *Eucalyptus* trees in Kenya for timber could be a significant factor in the frequency of *C. gattii* in Kenya (21).

Our findings indicate a decrease in susceptibilities among *C. neoformans*-*C. gattii* species complex to commonly used anti-fungal drugs (Table 1- 3). All the environmental and clinical *C. neoformans* isolates demonstrated high susceptibility to fluconazole (Table 1). Only one isolate each of *C. neoformans* (Table 1) from the clinical and environmental source was resistant to FLC. All the isolates displayed MICs (MIC ^{50} and MIC ^{90}) between 4 and 8 μg/ml to FLC which is on the susceptible range. This was lower than that reported by Bii *et al* (18) who also reported high MICs and resistance of 11.3% to FLC. Reduced susceptibilities to FLC have also been reported in other sub-Saharan African countries (11, 23). Study reports from other parts of the world have also shown increasing trend towards FLC resistance, for instance in Cambodia and Singapore; approximately 20% of clinical isolates were found to exhibit decreased susceptibility to FLC (24,25). Other studies have however detected no resistance to FLC. *C. neoformans* isolates from the United States, Thailand, and Malawi showed no significant difference in their susceptibility to fluconazole($p>0.05$) with susceptibility range of 1-32μg/ml contrary to our findings (26). These were on the range of susceptible ($\leq$8μg/ml) to susceptible dose dependent range (16-32 μg/ml), with no resistance reported. In Kenya, FLC is widely used for treatment of cryptococcal meningitis through the Diflucan Partnership Programme (14). In our study all *C. gattii* were fully susceptible to fluconazole. Elsewhere, reports of *C. gattii* anti-fungal susceptibility profile have been contradictory (27,28). A number of studies have found *C. gattii* to be less susceptible than *C. neoformans* to azole drugs, particularly FLC (29, 30). For instance a Brazillian susceptibility study by Trilles *et al*., in 2004 revealed less susceptibility to FLC among *C. gattii* with MIC range (8-64μg/ml) compared to *C. neoformans* with MIC range4-64 μg/ml to fluconazole (31).In adifferent study in Spain by Gomez *et al* 2008(30), poor activity to fluconazole, with MIC values higher than 4 μg/ml for 21 of 23 *C. neoformans* isolates (91%) was recorded. Although anti-fungal drug resistance of *C. neoformans* is rare worldwide with few isolated cases, emerging anti-fungal drug resistance should be monitored among the yeasts.We did not find any statistically significant differences in susceptibility between clinical and environmental *C. neoformans* ($P>0.05$). Our results are in agreement with those obtained by other authors (27, 32), who also demonstrated that anti-fungal susceptibility is not dependent on the origin of the isolates tested.

In our study all the clinical and environmental *C. neoformans* and *C. gattii* isolates were highly susceptible to AMP with MIC range of $\leq$0.25-8 μg/ml) (Table 2). Higher MICs was detected as compared to findings by Bii *et al*., (18) but no resistance was detected. This is probably because of the high cost of AMP and the need for parenteral administration that discourages its irrational use. Previous reports in Kenya and other parts of the world have revealed high susceptibilities of *C. neoformans* isolates to amphotericin B (18,19, 33). Studies by Franzot and Hamdan (34) and Yildiran *et al*.,(35) also displayed similar results with our findings with MIC range 0.125–1μg/ml in both cases. Contrary to our findings resistance to AMP have been detected using antibiotic medium 3 in a previous study (36).Other studies have also shown resistance among clinical *C. neoformans* isolates to AMP and therefore a need for its continuous surveillance for anti-fungal resistance (23, 37).We did not find any statistically significant differences in susceptibility between all clinical and environmental *C. neoformans* and *C.gattii* ($P>0.05$). In general 89/107 (83.2%) and 14/16 (87.5%) *C.neoformans* and *C.gattii* isolates respectively were susceptible to AMP with no significant difference in their susceptible range($P>0.05$).

Resistance to 5FC by clinical *Cryptococcus neoformans* was high as compared to the other drugs which were used in the study (Table 3). Despite the resistance exhibited by 5FC, we could not link susceptibility and clinical data to ascertain whether patients infected with these resistant strains had poor prognosis due to ethical reasons.The clinical isolates displayed higher MICs than environmental isolates probably due to induced resistance from previous anti-fungal exposure of patients during therapy. In many countries, 5FC is not indicated alone for the treatment of cryptococcosis due to treatment induced resistance (13,38). A recent susceptibility study of *Cryptococcus neoformans* isolates from cerebrospinal fluid (CSF) of HIV patients from Kenyatta National Hospital and Mbagathi District Hospital showed no resistance to 5FC. This was probably because all the isolates used were recovered from patients not previously exposed to anti-fungal drugs(33). Contrary to our results, a study by Trilles *et al*., (31) did not detect any resistance to 5FC with MIC of between

0.125-1 μg/ml. There were no significant differences in susceptibility of clinical and environmental isolatesto 5FC. However MIC $_{50}$ and MIC $_{90}$ of clinical isolates were higher as compared to that of environmental isolates.The MIC 50 and MIC 90 were 8 μg/ml and 64 μg/ml respectively which is in agreement with Bii *et al* (18) findings whereby an unusually high resistance rate of 21% was reported. In general 23/107 (21.5%) *C. neoformans* and 9/16 (56.3%) *C. gattii* isolates were susceptible to 5FC, while 75/107 (70.1%) and *C. gattii* 7/16 (43.8%) were at susceptible dose dependent range. There was a significant difference in 5FC susceptibility ranges between the two species; susceptible (P=0.003), and susceptible dose dependent range (S-DD), (P=0.038). Elsewhere, data on susceptibility to AMB and 5FC are more varied, with comparisons indicating that *C. gattii* is more resistant (39), more susceptible (30), or not different from (40,41). A possible reason for these findings is that noneof the studies considered the effect of genotypic and geographicdifferences within the two *Cryptococcus* species. The differences in ecology, epidemiology, and virulence,(42,43) and the regional differences (44) among cryptococcal genotypes are likely to reflect fundamental differences in their biology and physiology, which could affect their response to anti-fungal drugs. Despite differences in the susceptibility profiles in various investigations there is need to constantly monitor anti-fungal susceptibility to detect any developing resistance.

In conclusion, our results showed reduced susceptibility among *C. neoformans*-*C. gattii* species complex to flurocytosine and fluconazole as compared to amphotericin B. We also found no significant differences in susceptibilities among clinical and environmental isolates. The need for anti-fungal drug resistance surveillance is important for the management of cryptococcosis.

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REFERENCES

1. Rex JH, Pfaller MA. Has Anti-fungal Susceptibility Testing Come of Age? Degree of Correlation between In Vitro and Invivo. *Clin. Infect. Dis*. 2002;**35**:982-989.
2. Johnson EM. Issues in anti-fungal susceptibility testing. *J. Antimicrob. Chemother.* 2008; 61 Suppl 1:i13-i18.
3. 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.
4. Pappas PG. Cryptococcal infections in non-HIV-infected patients. *Trans. Am. Clin. Climatol. Assoc.* 2013;**124**:61-79.
5. Buchanan KL, Murphy JW. What makes *Cryptococcus neoformans* a pathogen? *Emerg. Infect. Dis*. 1998;**4**:71-83.
6. Kwon-chung KJ, Boekhout T, Fell JW, Diaz M. Proposal to conserve the name *Cryptococcus gattii* against *C . honduri anus* and *C . bacillisporus* (Basidiomycota, Tremellomycet- idae). *Taxon* 2002;**51**:804-806.
7. Galanis E. Epidemiology of *Cryptococcus gattii*, British Columbia, Canada, 1999–2007. *Emerg. Infect. Dis.* 2010;**16**:251-257.
8. Kidd SE, Chow Y, Mak S, *et al*. Characterization of Environmental Sources of the Human and Animal Pathogen *Cryptococcus gattii* in British Columbia, Canada, and the Pacific Northwest of the United States. *Appl. Environ. Microbiol.* 2007;**73**:1433-1443.
9. Litvintseva AP, Mitchell TG. Population genetic analyses reveal the African origin and strain variation of *Cryptococcus neoformans* var. *grubii*. *PLoS Pathog*. 2012;**8**:e1002495.
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.
11. Bicanic T, Harrison TS. Cryptococcal meningitis. Br. Med. Bull. 2004;72:99-118. doi:10.1093/bmb/ldh043.
12. Gordon SB, Walsh AL, Chaponda M *et al*. Bacterial meningitis in Malawian adults: pneumococcal disease is common, severe, and seasonal. *Clin. Infect. Dis.* 2000;**31**:53-57.
13. Nussbaum JC, Jackson A ND *et al.* Combination flucytosine and high dose fluconazole is superior to fluconazole monotherapy for cryptococcal meningitis: a randomized trial in Malawi. *Clin Infect Dis* 2010;**50**:338–344.
14. Diflucan Partnership Program. http://www.diflucanpartnership.org/en/welcome/.
15. Ferreira AS, Sampaio A, Maduro AP, *et al.* Genotypic diversity of environmental *Cryptococcus neoformans* isolates from Northern Portugal. *Mycoses* 2014;**57**:98-104.
16. EUCAST Definitive Document EDef 7 . 1: method for the determination of broth dilution MICs of anti-fungal agents for fermentative yeasts. 2008.
17. EUCAST DEFINITIVE DOCUMENT EDef 7.2 Revision Method for the determination of broth dilution minimum Inhibitory concentrations of anti-fungal agents for yeasts. 2008:1-21.
18. Bii CC, Makimura K, Abe S, *et al*. Anti-fungal drug susceptibility of *Cryptococcus neoformans* from clinical sources in Nairobi , Kenya. *Mycoses* 2006:25-30.
19. Tangwattanachuleeporn M, Weig M, Bader O. Prevalence and Anti-fungal Susceptibility of *Cryptococcus neoformans* Isolated from Pigeon Excreta in Chon Buri Province , Eastern Thailand. 2013;**54**.
20. Cogliati M. Global Molecular Epidemiology of *Cryptococcus neoformans* and *Cryptococcus gattii:* An Atlas of the Molecular Types. Scientifica (Cairo). 2013;2013(serotype D):675213.
21. Kangogo M, Boga H, Wanyoike W, Bii C. Isolation and Characterization of *Cryptococcus neoformans* and *Cryptococcus gattii* from Environmental Sources in Nairobi Kenya. *East Africa Med. J.* 2014;**91**:1-5.

22. Girish Kumar CP, Prabu D, Mitani H, Mikami Y, Menon T. Environmental isolation of *Cryptococcus neoformans* and *Cryptococcus gattii* from living trees in Guindy National Park, Chennai, South India. *Mycoses* 2010;**53**:262-264.
23. Neil F, Katherine G WC *et al*. *Cryptococcus* infection in a cohort of HIV-1-infected Ugandan adults. *AIDS* 2000;**16**:1031-1038.
24. Sar B, Monchy D, Vann M, Keo C, Sarthou J L A, 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.
25. KS: TA and C. *In vitro* activities of anti-fungal drugs against yeasts isolated from blood cultures and moulds isolated from various clinically significant sites in Singapore. *Ann Acad Med Singapore* 2008;**37**:841-846.
26. Archibald LK, Tuohy MJ, Wilson D a., *et al.* Anti-fungal Susceptibilities of *Cryptococcus neoformans*. *Emerg. Infect. Dis*. 2004;**10**:143-145.
27. Moraes, E. M. P., N. S. Prímola and JSH. Anti-fungal susceptibility of clinical and environmental isolates of *Cryptococcus neoformans* to four anti-fungal drugs determined by two techniques. *Mycoses* 2002;**46**:164-168.
28. NoYee-Chun, C., C. Shan-Chwen, S. Chiang-Ching, H. Chien-Ching LK-, Tay, P. Yueh-Shya and HW-C. Clinical features and in vitro susceptibilities of the two varieties of *Cryptococcus neoformans* in Taiwan. *Diagn. Microbiol. Infect. Dis.* 2000;**36**:175-183.
29. De Bedout, C., N. Ordonez, B. L. Gomez, M. C. Rodriguez, M. Arango A, Restrepo and EC. *In vitro* anti-fungal susceptibility of clinical isolates of *Cryptococcus neoformans* var. *neoformans* and *C. neoformans* var. *gattii*. *Rev. Iberoam. Micol.* 1999;**16**:36–39.
30. A. Gomez-Lopez, O. Zaragoza M Dos, Anjos Martins MCM, And JLR-T, Cuenca-Estrella1 M. *In vitro* susceptibility of *Cryptococcus gattii* clinical isolates. *Clin Microbiol Infect* 2008;**14**:727-730.
31. Trilles L, Fernandez-Torres B, Lazera Mdos S, Wanke B, Guarro J. *In vitro* anti-fungal susceptibility of *Cryptococcus gattii*. *J. Clin. Microbiol.* 2004;**42**:4815-4817.
32. Franzot, S. P. and JSH. *In vitro* susceptibilities of clinical and environmental isolates of *Cryptococcus neoformans* to five anti-fungal agents. *Antimicrob. Agents Chemother.* 1996;**40**:822–824.
33. Mdodo R, Moser SA, Jaoko W, *et al*. Anti-fungal susceptibilities of *Cryptococcus neoformans* cerebrospinal fluid isolates from AIDS patients in Kenya. 2012;**54**:1-7.
34. Franzot SP, Hamdan JS. *In vitro* susceptibilities of clinical and environmental isolates of *Cryptococcus neoformans* to five anti-fungal drugs. *Antimicrob. Agents Chemother.* 1996;**40**:822-824.
35. Yildiran, S. T., M. A. Saracli, A. W. Fothergill and MGR. *In vitro* susceptibility of environmental Cryptococcus variety neoformans isolates from Turkey to six anti-fungal agents, including SCH56592 and voriconazole. *Eur. J. Clin. Microbiol. Infect. Dis.* 2000;**19**:317–319.
36. Paetznick VL, Ghannoum MA. Detection of Resistance to Amphotericin B among *Cryptococcus neoformans* Clinical Isolates: Performances of Three Different Media Assessed by Using E-Test and National Committee for Clinical Laboratory Standards M27-A Methodologies. 1998;**36**:2817-2822.
37. Powderly WG, Keath EJ S-AM *et al*. Amphotericin B-resistant *Cryptococcus neoformans* in a patient with AIDS. *Infect Dis Clin* 1992;**1**:314-316.
38. Diamond DM, Bauer M, Daniel BE *et al*. Amphotericin B colloidal dispersion combined with flucytosine with or without fluconazole for treatment of murine cryptococcal meningitis. *Antimicrob Agents Chemother* 1998;**42**:528-533.
39. Chen, Y. C., S. C. Chang, C. C. Shih, C. C. Hung, K. T. Luhbd, Y. S. Pan A, Hsieh. WC. Clinical features and in vitro susceptibilities of two varieties of *Cryptococcus neoformans* in Taiwan. *Diagn. Microbiol. Infect. Dis.* 2000;**36**:175-183.
40. Trilles L, Lazéra MDS, Wanke B, *et al*. Regional pattern of the molecular types of *Cryptococcus neoformans* and *Cryptococcus gattii* in Brazil. *Mem. Inst. Oswaldo Cruz* 2008;**103**:455-462.
41. Thompson, G. R., III, N. P. Wiederhold, A. W. Fothergill, A. C. Vallor BL, Wickes and TFP. Anti-fungal susceptibilities among different serotypes of *Cryptococcus gattii* and *Cryptococcus neoformans*. *Antimicrob. Agents Chemother* 2009;**53**:309-311.
42. Ngamskulrungroj, P., F. Gilgado, J. Faganello, A. P. Litvintseva ALL, K. M. Tsui, T. G. Mitchell, M. H. Vainstein and WM. Genetic diversity of the *Cryptococcus* species complex suggests that *Cryptococcus gattii* deserves to have varieties. *PLoS One* 2009;**4**:e5862.
43. Bovers M, Hagen F, Boekhout T. Diversity of the *Cryptococcus neoformans-Cryptococcus gattii* species complex. *Rev. Iberoam. Micol.* 2008;**25**:S4-S12.
44. Fraser, J. A., S. S. Giles, E. C. Wenink, S. G. Geunes-Boyer, J. R. Wright S, Diezmann, A. Allen, J. E. Stajich, F. S. Dietrich, J. R. Perfect and JH. Same-sex mating and the origin of the Vancouver Island *Cryptococcus gattii* outbreak. *Nature* 2005;**437**:1360-1364.