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Multi-locus sequence typing reveals genotypic similarity in Nigerian Cryptococcus neoformans AFLP1/VNI of environmental and clinical origins

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Dear Prof. dr. Kidd,

Hereby we submit our revised manuscript entitled ‘Multi-locus sequence typing reveals genotypic similarity in Nigerian *Cryptococcus neoformans* AFLP1/VNI of environmental and clinical origins’ for your consideration for publication in the Journal of Medical Microbiology. The manuscript is co-authored by Paul E. Chidebelu, Emeka I. Nweze, Jacques F. Meis, Massimo Cogliati, and Ferry Hagen. All authors have seen and approved the manuscript and agreed with the content. Results reported are not published or considered for publication elsewhere.

We have adapted the manuscript according to the reviewer’s comments. A rebuttal letter with a point-by-point feedback is separately provided.

We hope that you find our study of interest to be published in the Journal of Medical Microbiology.

We look forward receiving your decision.

Yours sincerely,

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Multi-locus sequence typing reveals genotypic similarity in Nigerian *Cryptococcus neoformans* AFLP1/VNI of environmental and clinical origins

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Abstract

Introduction: Pigeon droppings are among the major environmental sources of *Cryptococcus neoformans* AFLP1/VNI, from where the organism infects susceptible humans and animals resulting in cryptococcosis. Until now, *C. neoformans* AFLP1B/VNII was the only molecular type reported in Nigeria. Effective clinical treatment of this infection has occasionally been stymied by the emergence of antifungal non-susceptible, and resistant strains of *C. neoformans* AFLP1/VNI.

Hypothesis/Gap statement: Pigeon droppings harbour *C. neoformans* and HIV/AIDS patients are among the susceptible population to develop cryptococcal infection. Epidemiological data on cryptococcal prevalence is limited in Nigeria.

Aim: To investigate the environmental prevalence of *C. neoformans* in South eastern Nigeria and compare the isolates with other lineages by using molecular and microbiological tools.

Methodology: A total of 500 pigeon droppings and 300 blood samples of HIV/AIDS patients were collected, respectively, from 5 market squares and 3 tertiary healthcare centers within the Nsukka area of South eastern Nigeria.The antifungal susceptibility of the *C. neoformans* isolates to amphotericin B, fluconazole, 5-fluorocytosine, itraconazole, voriconazole, posaconazole, and isavuconazole was investigated based on the CLSI M27-A3 protocol. Yeasts were identified by MALDI-TOF MS, thereafter *Cryptococcus* MLST was performed according to the International Society for Human and Animal Mycology (ISHAM) consensus scheme.

Results: *C. neoformans* was recovered from 6 (1.2%) pigeon droppings and 6 (2%) blood cultures of HIV/AIDS patients. Molecular analyses indicated that all cryptococcal isolates belong to serotype A and the AFLP1/VNI molecular type with sequence type (ST) 32. Infection with *C. neoformans* was independent of sex and age of the patients investigated. All *C. neoformans* isolates were susceptible to the seven antifungal agents.

61 **Conclusion:** This is the first report on the prevalence of *C. neoformans* AFLP1/VNI (ST32) in
62 environmental and clinical samples from Nigeria. The antifungal susceptibility indicates that
63 antifungal resistance by *C. neoformans* is yet a rare occurrence in Nigeria.

Introduction

Cryptococcus neoformans is the main etiologic agent of cryptococcosis and this basidiomycetous yeast can be morphologically described as an encapsulated, round to oval yeast cell measuring 2–7µm in diameter (1, 2). Its major virulence factors include among others, the large polysaccharide capsule, melanin pigments, and secretion of extracellular enzymes (2). Seven species within the *C. neoformans/C. gattii* species complexes have been recognized, being *C. neoformans* (serotype A; genotype AFLP1/VNI, AFLP1A/VNB/VNII and AFLP1B/VNII), *C. deneoformans* (serotype D; genotype AFLP2/VNIV), *C. gattii sensu stricto* (serotype B; genotype AFLP4/VGI), *C. bacillisporus* (serotype B and C; genotype AFLP5/VGIII), *C. deuterogattii* (serotype B; genotype AFLP6/VGII), *C. tetragattii* (serotype C; genotype AFLP7/VGIV), *C. decagattii* (serotype B; genotype AFLP10/VGIII and VGIV) (2).These species are widely distributed across different countries and different regions (1,2)

Pigeon droppings are among the major environmental sources of the pathogen (2-4), and as its mode of transmission, the aerosolized, desiccated basidiospores in the environment are usually acquired through inhalation (5). Predisposition to the infection is determined by certain underlying medical conditions including HIV/AIDS, solid organ transplant, diabetes, hepatic cirrhosis, haematological malignancies, tobacco smoking, among others (6). Consequently, the increased global burden of HIV/AIDS has spurred a concomitant surge in the emergence and spread of opportunistic infections including cryptococcosis (1). It is one of the leading opportunistic infections associated with HIV/AIDS, and 223,100 new cryptococcal meningitis cases are estimated to occur annually (7). In HIV/AIDS endemic settings, the infection is life-threatening where it causes cryptococcal meningitis, and other fulminant infections with a very high (40%) morbidity and mortality rate (8).

Since the endemicity of HIV/AIDS is high in Africa (9), environmental and clinical investigations remain necessary in order to understand the epidemiological patterns of cryptococcal infection. Molecular techniques such as PCR fingerprinting (1), *URA5*-RFLP (10), multi-locus sequence typing (MLST) (11), amplified fragment length polymorphisms (AFLP) analysis(2), and microsatellite typing (12) have been adopted for differentiation and confirmation of the various cryptococcal species/molecular types and isolates. Studies have shown that matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI–TOF MS) is a powerful tool for routine identification (13) and typing (14, 15) of pathogenic yeasts. Similarly, among the various molecular identification protocols, MLST is the preferred method for *Cryptococcus* genotyping (16).

In Sub-Saharan Africa, the annual mortality due to cryptococcosis is reportedly between 93,900 and 163,900(2). Concurrently, the sub-region records the greatest burden of cryptococcal meningitis constituting 50–70% globally (17). Comparing the 25 African countries already studied for occurrence of *C. neoformans* and *C. gattii* isolates, South Africa was found to have the highest prevalence rate of about 79% (1). Despite this, epidemiological information on clinical and environmental cryptococcal isolates remain obscure in many parts of Africa (18,19), including Nigeria (20,21). Also, the overt inconsistency in antifungal susceptibility profile complicated with constant development and acquisition of antimicrobial resistance by many clinical pathogens, has continued to generate speculation on the emergence of antifungal non-susceptible strains of *C. neoformans* (22). Paradoxically, the geometric rise in the immunodeficiency conditions especially that due to HIV/AIDS (7) is yet unparalleled with ample epidemiological data on antifungal susceptibilities of clinical and environmental strains of *C. neoformans* in many parts of Africa, which has further reinforced the rising index of morbidity from cryptococcosis (17). These epidemiological concerns made it necessary to investigate the prevalence of *C. neoformans* in both pigeon droppings and among HIV/AIDS

patients within our study area, and also study the *in vitro* susceptibility pattern of isolates to seven antifungal drugs.

Material and methods

Study area background

Nsukka is a geo-political zone in Enugu state of Southeast Nigeria. The area has a latitude of 6º51'24''N and a longitude of 7º 23'45''E and covers about 45.38 km^2 with the approximate elevation of 422.94 m. Most of the communities within the area practice agriculture including poultry farming as their major occupation.

Environmental samplings

Environmental samplings were performed in five major local markets chosen within the Nsukka geo-political area, where trading, handling, and exposure to various breeds of birds such as pigeons is a common practice. A total of 500 pigeon dropping samples were collected (100 from each market) using sterile collection tubes, from the following market squares: Ikpa Commodity Market (IM) in Nsukka local government area (LGA); OrieOrba Market (OOM) in Udenu LGA; AforObollo-Afor (AOA) in Udenu LGA; NkwoIbagwa Market (NI) in Igboeze South LGA; Eke Enugwu-Ezike Market (EEM) in Igboeze North LGA.

The pigeon droppings were weighed 5 g each into 50 mL of normal saline solution (1: 10) supplemented with 0.05 g/L chloramphenicol, and vortexed. The mixture was allowed to stand for about 10 min, and inoculated in aliquots of 100 µL into Sabouraud dextrose agar (SDA) (Titan Biotec, Delhi, India) supplemented with 0.05 g/L chloramphenicol. Suspected yeast colonies were subcultured onto modified caffeic acid agar (23). All the cultures were incubated at 37°C for up to 1 week.

Blood samples from HIV/AIDS patients

Clinical surveillance of cryptococcosis was carried out in tertiary healthcare institutions of Nsukka with facilities for the management of HIV/AIDS condition. Blood samples (5 mL) were collected from individuals who tested positive for HIV/AIDS and were also undergoing treatment in the facilities, using sterile blood culture containers (sterile McCartney bottles containing biphasic brain heart infusion/agar media with addition of 0.6 mg/ml polyanionic anticoagulant and sodium polyanethosulfonate). The ratio of blood to broth was 1:10, cultures were incubated aerobically at 37°C for up to 3 weeks. Information on the demography and the CD4 counts of the study population was also obtained

Characterization and identification of the C. neoformans/C. gattii *species complex isolates*

Cryptococcus neoformans/C. gattii species complex isolates were phenotypically identified by macroscopic and microscopic examination of yeast colonies, urease activity test, inositol assimilation, growth at 37°C, and melanin production on both the modified tobacco and caffeic acid agar culture media (23). The selected fresh colonies for identification were those showing the characteristic dark brown pigmentation on the agar media, and also indian ink positive on microscopy.

Species identification by MALDI-TOF MS

Isolates were grown on Sabouraud dextrose agar for 48 h and whole cell proteins were extracted using the ethanol-formic acid extraction method as described before (15). Measurements were performed with a Microflex LT mass spectrometer (Bruker Daltonics). Spectral processing and identification were performed using MALDI-Biotyper 3.0 software

(Bruker Daltonics). For each tested isolate, using the MALDI-Biotyper automation control software version 2.0.43.8 (Bruker Daltonics), a composite of six spectra was generated, resulting in a main spectrum (MSP), which contains the frequencies of the most significant peaks, average mass and intensity. The MSP of each isolate was used for pattern matching against the extended Biotyper 3.0 library database and an in-house MSP library entries consisting of 20 *Cryptococcus* isolates for each major molecular type (160 MSP). Identification scores were generated using the Biotyper 3.0 software (Bruker Daltonics). Values of 2.300–3.000 are rated as secure genus identification with probable species identification.

Multi-locus sequence typing (MLST) and evolutionary studies

MLST was performed according to the International Society for Human and Animal Mycology (ISHAM) consensus scheme (16). Genomic DNA was extracted as previously described (24). Amplification of the seven genetic loci and the subsequent sequencing analysis was done as described before (25).

Studies on the regional distribution of the isolates and sequence types of *C. neoformans* across Africa was done as described before (26). Allele type (AT), for each locus, and sequence type (ST), for each strain, were assigned comparing the sequences to those present in the global MLST database (11). The ST of the Nigerian isolates was then compared to a set of 111 STs from all African strains reported in the literature so far (27-30). A 1,000 bootstrapped unrooted maximum likelihood phylogenetic tree was inferred using MEGA v6.06 software (31) based on the alignment of 111 concatenated sequences of the seven MLST loci. The ATs combinations of the same set of African strains were used to infer a minimum spanning tree by goeBurst algorithm in Phyloviz software (32). A clonal complex (CC) was defined as a group containing STs with single or double locus variants.

Antifungal susceptibility testing

Susceptibilities of the *C. neoformans* isolates to seven antifungal drugs were done using the reference CLSI M27-A3 protocol for broth dilution antifungal susceptibility testing of yeasts (33). The included antifungal drugs were amphotericin B (Bristol – Meyers Squibb, Epernon, France), posaconazole (Schering-Plough Research Institute, Kenilworth, NJ, USA), isavuconazole (Basilea Pharmaceutica, Basel, Switzerland), itraconazole(Janssen Research Foundation, Beerse, Belgium), 5-fluorocytosine (F. Hoffmann – La Roche, Basel, Switzerland), fluconazole, and voriconazole (Pfizer, New York, NJ, USA). *Candida parapsilopsis* (ATCC 22019 = CBS 604) was used as a control strain.

Results

Isolates from pigeon droppings and blood samples

Six *C. neoformans* species complex isolates were recovered out of the 500 pigeon droppings collected from the five market squares. Three out of 100 samples from NIM, 2/100 samples from EEM and 1/100 from AOM yielded *C. neoformans* growth (Table 1). Samples taken at markets ICM and OOM remained negative. *Cryptococcus neoformans* was also identified in six out of the 300 blood samples of HIV/AIDS patients (Table 2).

Molecular type of the isolates

Identification by MALDI-TOF MS confirmed that the clinical and environmental isolates were *C. neoformans sensu stricto*. Further molecular characterisation by using MLST showed that they all belonged to molecular type AFLP1/VNI and genotype ST32 (GenBank

accession numbers LC338036-LC338042). The phylogenetic tree summarizes the evolutionary relatedness of the isolates with similar strains previously characterized (Figure 1). A total of 111 sequence types (STs) of *C. neoformans* were reported across the African continent so far. The majority of African STs are grouped in a large clade that includes only STs that belong to molecular type AFLP1/VNI (59 STs), a second well-defined clade include most of the VNII STs (12 STs), whereas VNB STs are not all grouped in a specific clade but share genetic similarities with some VNI STs. Some STs (NC5, NC17, ST210, ST263, ST249, ST221, and ST222) seems to be genetically distant from the clades described above. In this current work, the ST32 belonging to the largest VNI clade, had also been found in four other African countries: South Africa, Uganda, Tanzania, and D. R. Congo.

Distribution of the genotypes based on minimum spanning tree identified eight clonal complexes (CCs) among African *C. neoformans* isolates (Figure 2). These include two CCs among VNI isolates, two CCs among VNB, two CCs among VNII. and two CCs containing both VNI and VNB isolates. The CC that contained the sequence type of Nigerian isolates (ST32) showed two major clonal lineages including ST93 (reported in Uganda and South Africa) and ST31 (reported in Botswana, South Africa, Uganda, and Malawi). Variants STs of ST32 are ST39, ST91, and ST92 were from Uganda, and ST3, ST199, and ST200 were from South Africa.

Antifungal susceptibility testing

Susceptibilities of the *C. neoformans* isolates to the seven antifungal agents tested are listed in Table 3. All *C. neoformans* isolates had wild-type susceptibility to the tested antifungal agents.

Discussion

Cryptococcus neoformans sensu stricto is globally the major cryptococcosis causing culprit (1,2). The present study reports novel epidemiological information on the prevalence of environmental and clinical isolates of *C. neoformans* in Nigeria. *Cryptococcus neoformans* was recovered from pigeon droppings with a frequency of 1.2% (*n*=6/500) in three out of the five markets investigated. A much higher isolation rate (22%) was reported in a related prevalence study involving environmental sample sources within Southeastern Nigeria(4). The prevalence of *C. neoformans* from HIV/AIDS patients whose blood samples were cultured was 2% (*n*=6/300). Few studies on the clinical isolates of *C. neoformans* in Nigeria had until now relied on screening for seroprevalence of cryptococcal antigens among HIV/AIDS individuals (34,35). This study was therefore the first successful attempt at recovering *C. neoformans* from blood cultures of these patients in Nigeria. Despite routine utilization of cultures, serological techniques such as the lateral flow assays could offer a more sensitive and faster diagnostic approach (36). Our results are consistent with an earlier observation by Oladele and colleagues(34) who also found a seroprevalence rate of above 1%. Our findings indicate that patients with very low $CD4^+$ counts of $\leq$200 cells/µL were more susceptible to *C. neoformans* infection than patients with higher $CD4^+$ counts, which is in agreement with other findings (37-39).

The ratio of male to female patients who were positive for *C. neoformans* infection was found to be 1:2. The apparent sex distribution of cryptococcosis may not reflect the comprehensive burden of the disease among HIV/AIDS patients in our study area, since there was unequal sampling between the two sexes. Although it has been suggested that a higher incidence of cryptococcal infections in men occur because of their higher risk of HIV infection (40), there was no significant correlation between cryptococcosis and patients' sex in concordance with a previous report (41).

The molecular analysis revealed that all 12 Nigerian *C. neoformans* isolates belong to the molecular type VNI and genotype ST32. This result emphasized the general observation indicating that ST32 is one of the most distributed sequence types worldwide (1,2). Although several studies have confirmed molecular diversity and sequence types of *C. neoformans* VNI in some African countries (28-30), this is the first study reporting the molecular characterization of *C. neoformans* VNI in Nigeria. Another related study showed the occurrence of *C. neoformans* molecular type VNII with genotype ST43 from pigeon droppings in Jos, plateau state of North central Nigeria (21,37). Because of the limited epidemiology of *C. neoformans* in Nigeria, it is difficult to attribute the distribution of molecular types to the environmental differences existing between the northern region, where molecular type VNII was reported(42), and the southern region investigated in the present study. The findings presented here corroborate earlier observations confirming that molecular type VNI is the most globally distributed in both environmental and clinical samples, and as such the most common cause of cryptococcosis among the vulnerable individuals (1,2). Furthermore, the study outcome apparently reinforces the earlier suggestion that the origin of the molecular type is traceable to Africa (28). The data from our molecular studies showed that the environmental and clinical isolates are of similar phenotype and genotype and thus, supporting a previous claim that *C. neoformans* infection is acquired by inhalation of basidiospores abundant in the environment (5). The phenotypic and genotypic similarity between the environmental and clinical isolates as reported in this work, could suggest that *C. neoformans* is common in pigeon droppings within our study area and may serve as a possible source of human infection consistent with a related report (26). However, microsatellite study of isolates from Cuba could not establish the link between the pigeon guano-derived *C. neoformans* isolates and those recovered from human infections and authors suggested the inclusion of other environmental sources (43,44). It also agrees with an observation from Vietnam that *C. neoformans* molecular type VNI was

281 responsible for infections in more than 70% of non-HIV and up to 100% HIV-infected patients
282 (45).. From the phylogenetic tree analysis for evolutionary relatedness, it is evident that the *C.*
283 *neoformans* molecular type VNI has not been reported previously from Nigeria.. Thus, this is
284 apparently the first from the sub-region, while the sequence type has been found in only four
285 other African countries, namely D.R. Congo, South Africa,Tanzania, and Uganda as recorded
286 in the ISHAM MLST database (http://mlst.mycologylab.org/). Epidemiological investigations
287 on the prevalence and transmission of cryptococcosis should therefore be of high considerations
288 especially in HIV/AIDS endemic regions.

289 There are sufficient reports on the susceptibility of clinical and environmental *C.*
290 *neoformans* isolates to many antifungal drugs (2,47). Previous observation also showed that
291 both clinical and environmental isolates exhibit similar pattern of susceptibility to antifungal
292 agents without any record of resistance (48). We tested the antifungal susceptibility of the
293 Nigerian *C. neoformans* isolates using a total of seven antifungal drugs including amphotericin
294 B, 5-fluorocytosine, fluconazole, itraconazole, posaconazole, voriconazole, and isavuconazole.
295 Interestingly, all isolates were susceptible to the antifungal drugs used.

296 Central to the treatment of cryptococcosis is amphotericin B used either singly or as a
297 combined amphotericin B and 5-fluorocytosine therapy, as the choice antifungal regimen
298 especially at the induction phase in cryptococcal meningitis (49). Rare occurrence of resistance
299 to amphotericin B among *C. neoformans* isolates may be one of the important considerations
300 for its consistent clinical use (50). They reported that both clinical and environmental
301 cryptococcal isolates are susceptible with equal MIC values when tested against amphotericin
302 B. This is even more evident in our present observation as we also indicated a low and equal
303 MIC value (0.25 µg/ml) of amphotericin B for both clinical and environmental isolates.
304 Although there is a sparse indication of elevated MICs of the drug when used against some

isolates (51), our result suggested similarity in antifungal susceptibility of the isolates to amphotericin B irrespective of source.

Within the clinical isolates, fluconazole, voriconazole, isavuconazole, and 5-fluorocytocine showed different values of MIC's against *C. neoformans* isolates. The results showed that fluconazole and 5-fluorocytocine have equal MIC range (2-4 µg/ml), while voriconazole and isavuconazole similarly showed equivalent MIC ranges of 0.031-0.063 µg/ml. This is consistent with previous work which showed equivalent, but narrower MIC ranges of 0.015-0.5 µg/ml for voriconazole, isavuconazole, and posaconazole (52).

Similarly, the susceptibility patterns of *C. neoformans* isolates from the environmental group to the antifungal agents tested showed a slight variation in MIC ranges of some of the antifungals with voriconazole and isavuconazole presenting the lowest MIC ranges (0.031-0.063 µg/ml). This has been corroborated in other studies suggesting that these drugs seem to offer treatment advantage over other antifungal drugs (53).

Antifungal susceptibility among *C. neoformans* isolates is not source-dependent as revealed by this present work and thus, supports the observation that no significant difference in antifungal susceptibility exists between the environmental and clinical isolates (48,54). Most of the newer azole derivatives unlike fluconazole, are either not available or yet to be approved for use in some settings, but it remains of high clinical relevance to investigate their efficiency and ease of use in the clinical management of cryptococcosis given the continuous observation of non-susceptible *C. neoformans* isolates (22). Our results have also helped to allay the fear of emerging resistant strains of *C. neoformans* within our study area, though can be envisaged but at least yet to be encountered.

Obviously, the newer azole antifungal agents such as voriconazole, isavuconazole, and posaconazole. which showed relatively high *in vitro* activities against *C. neoformans*, can serve

as excellent and cheaper alternatives that can be included in the treatment of cryptococcosis in resource-limited settings particularly. This is necessary to put in check the possible emergence of antifungal resistant *Cryptococcus* strains, especially in HIV/AIDS endemic regions, where cryptococcosis threatens the clinical outcome and survival of the victims.

Conclusion

Pigeon droppings are a major environmental source for *C. neoformans* in the study area. With extensive environmental studies of other samples in the study area, identification of sequence types other than ST32, is likely. We did not observe antifungal resistance, continuous clinical surveillance and adherence to antifungal therapy administration guidelines are key to sustaining the antifungal susceptibility profile of this pathogen.

Author's contributions

Conceptualisation: PEC, EIN, JFM, MC, FH; Methodology: PEC, EIND, MC, FH; Formal analysis: PEC, MC, FH; Investigation: PEC, EIN, JFM, MC, FH; Resources: EIN, JFM, MC, FH; Data curation: EIN, MC, FH; Writing: PEC, EIN, JFM, MC, FH.

Conflicts of interest

None of the authors declared a conflict of interest.

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Ethical Approval

Ethical approval from the Research and Ethics Committee (REC) of Bishop Shanahan Hospital Nsukka, EnugwuEzike District Hospital, and Nsukka District Hospital, all within Nsukka study area, Enugu State Nigeria, were obtained with respective reference numbers 19/01/2015-20/02/2015; 02/03/2015 - 01/04/2015; and 04/05/2015 - 06/06/2015. Informed consent of the volunteered study population were sought prior to their enrolment. Throughout the study period, utmost confidentiality was observed in handling and analysis of the research data.

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Figure legends

Figure 1. Unrooted maximum likelihood phylogenetic tree inferred using MEGA v6.06 software (27) based on the alignment of 111 concatenated sequences of seven MLST loci (*CAP59*, *GPD1*, IGS1, *LAC1*, *PLB1*, *SOD1*, and *URA5*). The analysis includes all*Cryptococcusneoformans* sequence types identified in Africa so far. Different colors of the squares indicate different molecular types. The red arrow indicates the position of ST32 in the phylogenetic tree. NC indicates genotypes described in the literature but not deposited in a public MLST database.

Figure 2. Minimum spanning tree inferred using goeBurst algorithm in Phyloviz software (28). Clonal complexes are grouped in solid lines and included sequence types that do not differ in more than two loci (double locus variant). Different colors of the solid lines indicate different molecular types. The number of locus variants is indicated on each linkage line. Each circle represents a single sequence type and its size is proportional to the number of strains with the same genotype. Different colors of the circles indicate different African countries.

566

567 Table 1: Environmental distribution of the *Cryptococcus neoformans* isolates

S/No	Site of sample collection	Number of samples collected	Number of samples positive (%)
1	Ikpa Commodity Market (ICM)	100	0 (0.00)
2	Nkwo Ibagwa Market (NIM)	100	3 (0.60)
3	Orie-Orba Market (OOM)	100	0 (0.00)
4	Afor-Obollo Market (AOM)	100	1 (0.20)
5	Eke Enugu Ezike Market (EEM)	100	2 (0.40)
	Total	500	6 (1.20)

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570

571 Table 2: Demography and CD4 counts of the sample population positive for *C.neoformans*

Age range (years)	Sample population (%)	Total number positive (%)	Sex	CD4 count (cells/mm^3)
< 9	24 (8.0)	1 (16.66)	F	51

10 – 19	18 (6.0)	0 (0)		
20 – 29	60 (20.0)	1 (16.66)	F	4
30 – 39	123 (41.0)	1 (16.66)	F	169
40 – 49	60 (20.0)	1 (16.66)	M	71
50 – 59	14 (4.7)	1 (16.66)	F	110
60 – 69	1 (0.3)	1 (16.66)	M	440
Total	300	6		

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573

574 **Table 3. Minimal inhibitory concentration (**μg/ml**) of clinical and environmental isolates.**

Isolates	**AMB**	**FLC**	**ITC**	**VOR**	**POS**	**ISA**	**5FC**
Env. 1	0.25	4	0.063	0.031	0.063	0.031	4
Env. 2	0.25	4	0.063	0.031	0.063	0.031	4
Env. 3	0.25	4	0.063	0.063	0.063	0.031	2
Env. 4	0.25	4	0.125	0.063	0.063	0.063	4
Env. 5	0.25	4	0.063	0.031	0.063	0.031	4
Env. 6	0.25	4	0.063	0.031	0.063	0.031	2
Clin. 7	0.25	2	0.063	0.031	0.063	0.031	2
Clin. 8	0.25	2	0.063	0.031	0.063	0.031	2
Clin. 9	0.25	4	0.063	0.031	0.063	0.031	2
Clin. 12	0.25	4	0.063	0.063	0.063	0.063	4
Clin. 13	0.25	4	0.063	0.063	0.063	0.031	4
Clin. 14	0.25	4	0.063	0.031	0.063	0.031	4

575 AMB = Amphotericin B, FLC = Fluconazole, ITC = Itraconazole, VOR = Voriconazole, POS
576 = Posaconazole, ISA = Isavuconazole, 5FC = 5-fluorocytosine.

577

578

Figure 1

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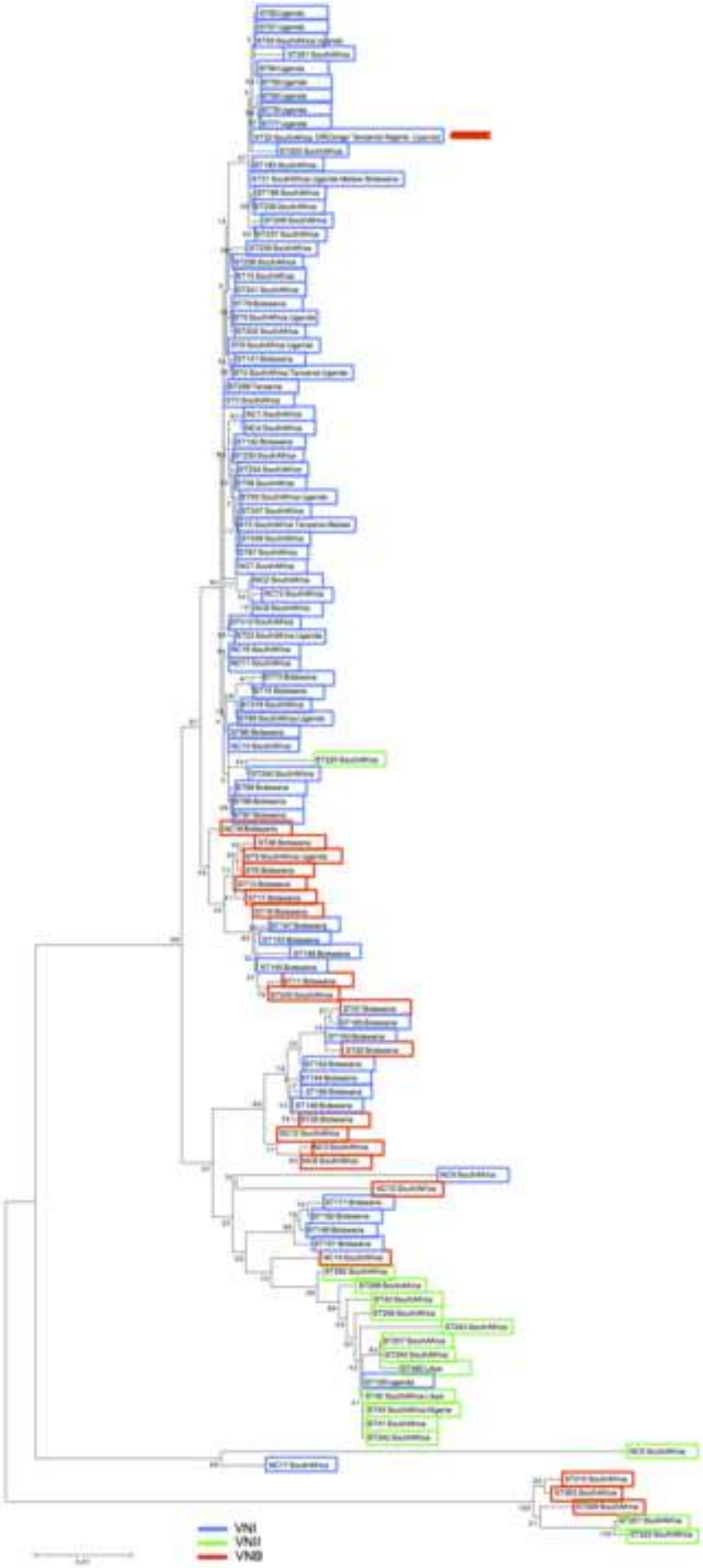

Figure 2

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