Cryptococcus neoformans population diversity and clinical outcomes of HIV-associated cryptococcal meningitis patients in Zimbabwe

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HIV and cryptococcal meningitis co-infection is a major public health problem in most developing countries. *Cryptococcus neoformans sensu stricto* is responsible for the majority of HIV-associated cryptococcosis cases in sub-Saharan Africa. Despite the available information, little is known about cryptococcal population diversity and its association with clinical outcomes in patients with HIV-associated cryptococcal meningitis in sub-Saharan Africa. In a prospective cohort, we investigated the prevalence and clinical outcome of *Cryptococcus neoformans sensu stricto* meningitis among HIV-infected patients in Harare, Zimbabwe, and compared the genotypic diversity of the isolates with those collected from other parts of Africa. Molecular typing was done using amplified fragment length polymorphism genotyping and microsatellite typing. The majority of patients with HIV-associated *Cryptococcus neoformans sensu stricto* meningitis in this cohort were males (n=33/55; 60.0 %). The predominant *Cryptococcus neoformans sensu stricto* genotype among the Zimbabwean isolates was genotype AFLP1/VNI (n=40; 72.7 %), followed by AFLP1A/VNB/VNII (n=8; 14.6 %), and AFLP1B/VNII was the least isolated (n=7; 12.7 %). Most of the isolates were mating-type α (n=51; 92.7 %), and only four (7.3 %) were mating-type **a**. Overall in-hospital mortality was 55.6 % (n=30), and no difference between infecting genotype and clinical outcome of patient (P=0.73) or $CD4^+$ counts (P=0.79) was observed. Zimbabwean *Cryptococcus neoformans sensu stricto* genotypes demonstrated a high level of genetic diversity

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Abbreviations: AFLP, amplified fragment length polymorphism; ART, antiretroviral therapy; CM, cryptococcal meningitis; HAART, highly active antiretroviral therapy; MC, microsatellite cluster.

One supplementary table is available with the online Supplementary Material.

by microsatellite typing, and 51 genotypes within the main molecular types AFLP1/VNI, AFLP1A/VNB/VNII and AFLP1B/VNII were identified. This study demonstrates that *Cryptococcus neoformans sensu stricto* in Zimbabwe has a high level of genetic diversity when compared to other regional isolates.

INTRODUCTION

The genus *Cryptococcus*, which belongs to the fungal phylum Basidiomycota, is polyphyletic and has been known for more than a century (Fonseca *et al.*, 2011; Liu *et al.*, 2015a, 2015b). *Cryptococcus neoformans sensu stricto* is ubiquitous in nature and can readily be isolated from air, soil and soils contaminated with pigeon guano and in avian habitats (Mitchell & Perfect, 1995). The HIV epidemic has raised the profile of *Cryptococcus neoformans sensu stricto* from a rare yeast to one of the most important fungal causes of morbidity and mortality worldwide. *Cryptococcus neoformans sensu stricto* is a major cause of HIV-associated cryptococcal meningitis (CM) globally (Hagen *et al.*, 2015; Nyazika *et al.*, 2016b).

Globally, an estimated 1 million cases of CM occur annually, with sub-Saharan Africa having the highest yearly burden with an estimate of 720 000 cases (Park *et al.*, 2009). Prior to upscaling of antiretroviral therapy (ART), an estimated annual mortality of 500 000 cases occurred in sub-Saharan Africa as a result of CM (Park *et al.*, 2009). Despite the increasing availability of ART, studies conducted in sub-Saharan Africa have demonstrated that acute mortality as a result of HIV-associated CM still remains above 40.0 % (Beardsley *et al.*, 2016; Boulware *et al.*, 2014; Kambugu *et al.*, 2008; Lessells *et al.*, 2011; Makadzange *et al.*, 2010; Nyazika *et al.*, 2016a).

Cryptococcus neoformans sensu stricto genotypes AFLP1/VNI and AFLP1B/VNII are widely distributed throughout the world, with AFLP1/VNI being the major cause of CM in HIV-infected individuals (Cogliati, 2013; Hagen *et al.*, 2015). The genotype AFLP1A/VNB/VNII appears to be endemic in Africa; there are increasing reports that it might have a global distribution (Cogliati, 2013). However, the epidemiology seems to be changing with the rise of *Cryptococcus gattii sensu lato* cases observed in some African studies (Litvintseva *et al.*, 2005; Nyazika *et al.*, 2016b). Studies conducted on the African continent have found some of the *Cryptococcus neoformans sensu stricto* genotypes and their mating types to be associated with clinical outcome in patients with HIV-associated CM (Beale *et al.*, 2015; Hagen *et al.*, 2015; Wiesner *et al.*, 2012). Despite the available information, little is known about *Cryptococcus neoformans* population diversity, prevalence and its association with clinical outcomes in patients with HIV-associated CM living in sub-Saharan Africa. Therefore, we investigated the genetic diversity of *Cryptococcus neoformans* isolates and clinical outcomes of Zimbabwean patients with HIV-associated CM. In addition, the genetic diversity of *Cryptococcus neoformans* from the current cohort was compared by microsatellite typing with that of isolates collected from other countries within the sub-Saharan Africa.

METHODS

Patients and isolates. A total of 100 consenting HIV-infected adult inpatients from Parirenyatwa Group of Hospitals (Harare, Zimbabwe) presenting with signs and symptoms of meningitis were enrolled into the cohort study between June 2013 and September 2014. Information on patient demographic data, clinical data and length of hospital stay and diagnostic test results was collected. Cerebrospinal fluid from patients was tested using a cryptococcal antigen lateral flow assay (IMMY Diagnostics) and Indian ink staining and plated onto Sabouraud dextrose agar supplemented with chloramphenicol (0.5 g l^{-1}) (Oxoid). Seventy-four cerebrospinal fluid samples were culture positive and were suggestive of *Cryptococcus* species after 7 days of incubation. These isolates were then subjected to biotyping, and 66 of 74 could be revived by the receiving laboratory for molecular typing.

Biotyping *of Cryptococcus* isolates. Two-day-old *Cryptococcus* isolates grown onto Sabouraud dextrose agar supplemented with 0.4 g chloramphenicol (Oxoid) were biotyped using L-canavanine glycine bromothymol blue, yeast carbon base D-proline D-tryptophan and creatinine dextrose bromothymol blue thymine media (all Sigma-Aldrich). The culture plates were incubated at 25 °C for 5 to 10 days. All three biotyping media were prepared according to previously described standard protocols (Chaskes *et al.*, 2008; Irokanulo *et al.*, 1994; Kwon-Chung *et al.*, 1982). *Candida albicans* ATCC 10231, *Candida parapsilosis* ATCC 22019 and *Cryptococcus neoformans* ATCC 204092 were used as quality control strains.

Genomic DNA extraction, amplified fragment length polymorphism genotyping and mating typing. Genomic DNA was extracted from 2-day-old sabouraud dextrose agar with chloramphenicol (SAB +C) cultures of *Cryptococcus* isolates. *Cryptococcus* cells were suspended in 400 µl bacterial lysis buffer followed by bead beating in Green Beads tubes with a MagNA Lyser for 30 s at 6500 r.p.m. (Roche Diagnostics). The lysed material was heat inactivated for 15 min at 100 °C, and 200 µl of the suspension was transferred to a 96 DeepWell plate. Automatic DNA extraction on a MagNA Pure 96 platform was performed using the Pathogen 200 protocol with a final elution volume of 100 µl (Roche Diagnostics).

Amplified fragment length polymorphism (AFLP) genotyping was performed on the *Cryptococcus* genomic DNA according to previously described protocols (Boekhout *et al.*, 2001; Illnait-Zaragozi *et al.*, 2010). The following reference strains were included in AFLP genotyping: 125.91 and H99 (both *Cryptococcus neoformans sensu stricto* AFLP1/VNI), Bt1 (*Cryptococcus neoformans sensu stricto* AFLP1A/VNB/VNII), WM626 (*Cryptococcus neoformans sensu stricto* AFLP1B/VNII), JEC20 and JEC21 (both *Cryptococcus deneoformans* AFLP2/VNIV), CBS132 and WM629 (both *Cryptococcus neoformans*×*Cryptococcus deneoformans* AFLP3/VNIII), WM179 (*Cryptococcus gattii sensu stricto* AFLP4/VGI), WM161 (*Cryptococcus bacillisporus* AFLP5/VGIII), WM178 (*Cryptococcus deuterogattii* AFLP6/VGII), WM779 (*Cryptococcus tetragattii* AFLP7/VGIV) and IHEM14941 (*Cryptococcus decagattii* AFLP10/VGIV) (Hagen *et al.*, 2012, 2015).

Mating type of the cryptococcal isolates was determined by using two multiplex PCRs that target the *RUM1* mating-type **a** and α alleles of *Cryptococcus neoformans* and *Cryptococcus deneoformans* (Arsic

Arsenijevic *et al.*, 2014). The *Cryptococcus neoformans sensu stricto* reference strains 125.91 (CBS 10512; aA, AFLP1/VNI) and H99 (CBS 8710; αA; AFLP1/VNI) and *Cryptococcus deneoformans* reference strains JEC20 (CBS 10511; aD; AFLP2/VNIV) and JEC21 (CBS 10513; αD; AFLP2/VNIV) were included as controls.

Microsatellite typing. The genetic relatedness of the *Cryptococcus neoformans* isolates was investigated using microsatellite typing as previously described by Illnait-Zaragozi *et al.* (2010). A set of 86 *Cryptococcus neoformans* isolates previously obtained from clinical, environmental and veterinary sources collected from neighbouring African countries, as well as from Belgian patients with cryptococcosis who visited African countries, were included (Table S1, available in the online Supplementary Material). Fragment analysis was performed on an ABI3500xL Genetic Analyser platform (Applied Biosystems) after the multiplex PCR amplicons were diluted 100 times, and 1 µl of this dilution was mixed with 0.1 µl LIZ600 (Applied Biosystems) and 8.9 µl ddH_2O followed by a 1 min heating step at 100 °C. Raw data were analysed with the GeneMapper software package v1.0 (Applied Biosystems), and the obtained microsatellite profiles were imported into Bionumerics v7.5 (Applied Maths); the data were treated as categorical values to generate a minimum spanning tree. The genetic diversity was calculated by using the Simpson's diversity index (D) that results in a value ranging from 0 to 1, indicating that either all isolates are the same (D=0) or all isolates are different (D=1) (Illnait-Zaragozi *et al.*, 2010). The index of association (I_A) and $\hat{r}_d$, indicators for the presence of clonality or recombination, were calculated by using Multilocus v1.3b program on a clone-corrected dataset and tested against 100 000 artificially recombined datasets (Agapow & Burt, 2001; Hiremath *et al.*, 2008).

Ethical approval. Institutional ethical approval for the study was obtained from the Joint Research Ethics Committee of the Parirenyatwa Group of Hospitals (Harare, Zimbabwe) and the College of Health Sciences, the Institutional Review Board of the Biomedical Research Training Institute, the Medical Research Council of Zimbabwe and Research Council of Zimbabwe. Informed written patient consent was obtained, and demographic data were collected. Patients were followed up until they were discharged or deceased.

Statistical analysis. Data were analysed as follows: categorical data (gender of the patient, *Cryptococcus neoformans* genotype and patient clinical outcome) were presented as frequencies, while continuous data (CD4 cell count, age of participants and duration of symptoms) were presented using means (SD) and median (interquartile range). Comparisons of $CD4^+$ count (continuous) by the *Cryptococcus neoformans* genotype and highly active antiretroviral therapy (HAART) status were analysed using Kruskal–Wallis ANOVA. Fisher's exact test statistic was used to compare clinical outcomes of patients infected with the different genotypes of *Cryptococcus neoformans sensu stricto*. Multivariate analysis was used to determine factors that were associated with the patient clinical outcome. All statistical analyses were performed using the Stata software v13 (StataCorp). $P<0.05$ was considered as statistically significant, and observations with missing values were excluded from the analysis.

RESULTS

Demographic characteristics of the study population

In a cohort of patients with HIV-associated *Cryptococcus neoformans sensu stricto* meningitis, the majority (n=33/55; 60.0 %) were males. The age of the patients ranged from 18 to 58 years with a median age of 36 years. All but one of the patients (n=54/55; 98.2 %) were admitted with headache and were treated empirically with 1 g ceftriaxone (n=51/54; 94.4 %) before final diagnosis of CM was made. This is summarized in Table 1.

Differences in $CD4^+$ counts between the HAART-naïve and -experienced patients

Among the patients with *Cryptococcus neoformans sensu stricto* meningitis, 54.5 % (n=30/55) were on HAART, and the rest were HAART naïve. Of the 55 patients, 47 had a $CD4^+$ cell count done. Forty-one (87.2 %) patients were severely immunocompromised, and their $CD4^+$ cell counts were $\leq$50 cells mm^{-3}. Two (4.3 %) patients had a $CD4^+$ cell count greater than 100, which is rare with HIV-associated CM, and there was no adequate clinical information to explain this phenomenon. The difference between median $CD4^+$ cell counts of patients with HIV-associated CM on HAART and those who were HAART naïve was not statistically significant (χ^2=0.078; P=0.78).

Prevalence of genotypes, mating type and association of genotype and $CD4^+$ count

Of the 74 isolates, 14 were identified to be *Cryptococcus gattii sensu lato* and 60 were identified to be *Cryptococcus neoformans sensu lato* on both L-canavanine glycine bromothymol blue and yeast carbon base D-proline D-tryptophan biotyping media. Sixty-six isolates were available for molecular analysis, as 8 isolates could not be revived by the receiving laboratory and included 55 (83.3 %) *Cryptococcus neoformans sensu lato* isolates and 11 (16.7 %) *Cryptococcus gattii sensu lato* isolates of which one isolate contained two phenotypic different colony types (Nyazika *et al.*, 2016a). Upon genotyping 55 of the *Cryptococcus neoformans sensu lato* isolates, *Cryptococcus neoformans sensu stricto* genotype AFLP1/VNI was the most prevalent (n=40; 72.7 %), followed by AFLP1A/VNB/VNII (n=8; 14.6 %) and AFLP1B/VNII (n=7; 12.7 %) (Fig. 1). The majority of the *Cryptococcus neoformans sensu lato* isolates were mating-type α (n=51; 92.7 %), and only four (7.3 %) were mating-type a. The in-depth molecular characterization of the *Cryptococcus gattii sensu lato* isolates has recently been described elsewhere (Nyazika *et al.*, 2016a). The median $CD4^+$ of patients with genotype AFLP1/VNI was 23 (12–35), genotype AFLP1A/VNB/VNII was 22 (13.5–36) and that of AFLP1B/VNII was 26 (15–66) cells mm^{-3}. There was no

Table 1. Patient demographic characteristics

Characteristic	Patients, n=55 (%)	Median (IQR)
Age (years)	54 (98.2)	36 (30–43)
Headache duration (days)	54 (98.2)	14 (7–21)
$CD4^+$ cell count (cells mm^{-3})	47 (87.5)	24 (12–40)
Weeks since HIV diagnosis	54 (98.2)	8 (2–104)
Hospital stay (days)	54 (98.2)	17.5 (10–22)

IQR, interquartile range.

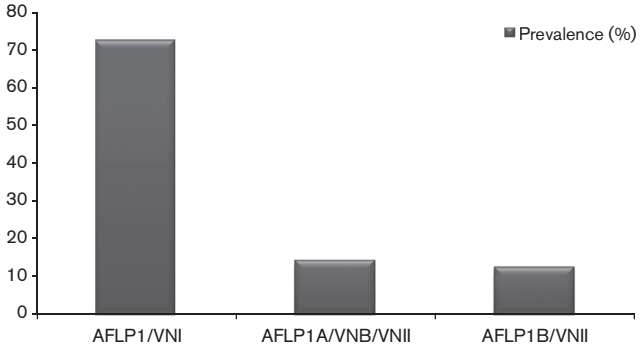

Fig. 1. Prevalence of isolated clinical *Cryptococcus neoformans sensu stricto* genotypes from Zimbabwe.

significant difference in $CD4^+$ count among patients infected with the different genotypes of *Cryptococcus neoformans sensu stricto* (χ^2=0.46; *P*=0.79).

The association of *Cryptococcus neoformans sensu stricto* genotype and clinical outcome of patients

The overall in-hospital mortality in the study was 30 (55.6 %) of 54; one patient was lost to follow-up. The in-hospital mortality for patients infected with genotype AFLP1/VNI was 22/39 (56.4 %); AFLP1A/VNB/VNII 5/8 (62.5 %); and AFLP1B/VNII 3/7 (42.9 %). The frequency distribution of cryptococcal genotype by patient outcome is summarized in Table 2. There was no significant difference between the genotypes and clinical outcome (χ^2=0.62; *P*=0.73). Using logistic regression analysis, factors associated with discharge of a patient were length of hospital stay (odds ratio, 1.09; 95 % confidence interval, 1.011–1.181) and being on HAART (odds ratio, 0.21; 95 % confidence interval, 0.047–0.95) after controlling for age and gender.

Genetic relatedness of Zimbabwean and other African *Cryptococcus neoformans sensu stricto* isolates

One hundred and forty-one *Cryptococcus neoformans* isolates were genotyped using AFLP and microsatellite typing methods. The relationship between different genotypes and sources is shown in Fig. 2. There was a large genotypic diversity among the different *Cryptococcus neoformans sensu stricto* isolated from various African countries. Genotypes were recognized and indicated as microsatellite complexes. Five microsatellite clusters (MCs) could be observed (Fig. 2a, indicated with a grey shadow), which contain at least three isolates that do not differ more than one of nine microsatellite loci from each other. The two largest MCs contain Zimbabwean isolates from the current study, as well as isolates obtained during a previous study in the 1990s. The largest MC contains isolates that have (nearly) identical microsatellite profiles but were obtained from different geographic sites (Fig. 2a). No statistically significant correlation was observed between microsatellite profiles and genotype AFLP1A/VNB/VNII or AFLP1B/VNII isolates, although the two largest MCs contain solely isolates with genotype AFLP1/VNI (Fig. 2b). Similarly, no statistically significant correlation was observed between microsatellite profiles and the background of the studied isolates; nevertheless, nearly all environmental isolates clustered together in the second-largest MC (Fig. 2c). The Simpson's diversity index was 0.9930 for all the African isolates (*n*=141) with 101 genotypes, and the Zimbabwean *Cryptococcus neoformans sensu stricto* had 51 genotypes present with a Simpson's diversity index of 0.9953 (Table 3). The observed values for the linkage disequilibrium index I_A and the less biased index $\hat{r}_d$ were found to be significant (*P*<0.0001); the H_0 hypothesis that there is no linkage among markers was rejected, which indicates a clonal population (Table 3).

Table 2. Distribution of *Cryptococcus neoformans* genotypes and outcome of patient

Cryptococcus genotype	Outcome		*P* value
	Death, *n* (%)	Discharge, *n* (%)	
AFLP1/VNI	22 (56.4)	17 (43.6)	0.73
AFLP1A/VNB/VNII	5 (62.5)	3 (37.5)	
AFLP1B/VNII	3 (42.9)	4 (57.1)	

χ^2=0.62.

Table 3. Simpson's diversity index and linkage disequilibrium

	$n_{isolates}$	$n_{genotypes}$	Simpson's *D*	Index of association (*P* value)	$\hat{r}_d$ (*P* value)	Isolates from study
Africa	141	103	0.9930	2.19535 (<0.0001)	0.297743 (<0.0001)	This study
Zimbabwe (current study)	55	51	0.9953	1.95722 (<0.0001)	0.267281 (<0.0001)	This study
D.R. Congo	20	12	0.9211	5.58276 (<0.0001)	0.798087 (<0.0001)	Swinne *et al.* (1986)
Rwanda	26	22	0.9846	3.79816 (<0.0001)	0.518182 (<0.0001)	Bogaerts *et al.* (1999)

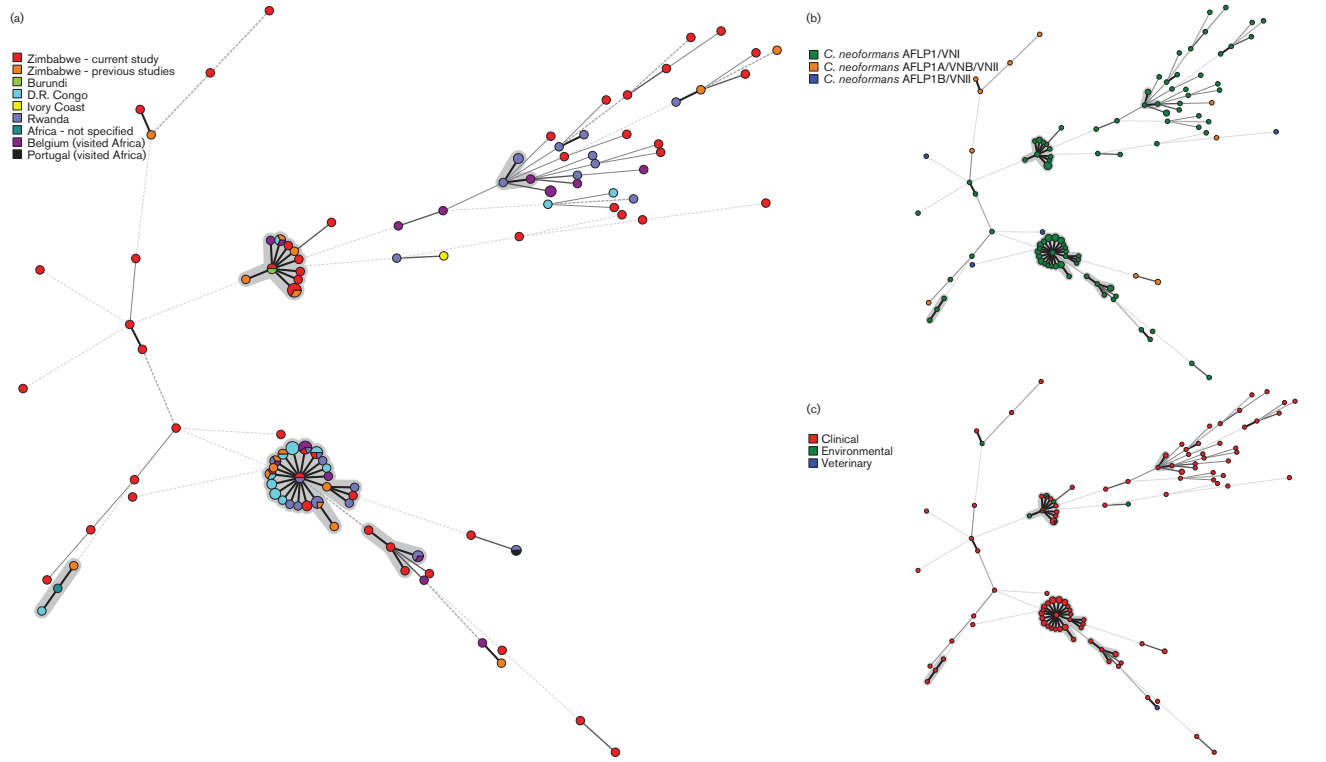

Fig. 2. (a) Minimum spanning tree (MST) analysis representing the genotypes of 141 *Cryptococcus neoformans sensu stricto* isolates from Africa collected between 1979 and 2014 based on a nine-locus microsatellite typing panel. Each circle represents a unique microsatellite genotype, and the size of the circle corresponds to the number of isolates within that genotype. Genotypes are linked to their closest relative with a line, being the thick black line (one-locus difference), thick dark grey line (two-locus difference), thick grey line (three-locus difference), thick grey dotted line (four-locus difference) and thin grey dotted line (difference greater than four loci). Genotypes connected by a shaded background are part of a microsatellite complex, only present within the *Cryptococcus neoformans* genotype AFLP1/VNI. (b) Same MST as in (a), but now showing the genotypic background of the studied isolates. (c) Same MST as in (a), but now showing the genotypes obtained from clinical, environmental and veterinary samples.

DISCUSSION

Cryptococcus neoformans sensu stricto is the most common causative agent of HIV-associated CM in people living in sub-Saharan African countries, where it accounts for more than 500 000 deaths per year before the upscaling of ART (Beale *et al.*, 2015; Kangogo *et al.*, 2015; Litvintseva *et al.*, 2005; Nyazika *et al.*, 2016b; Park *et al.*, 2009). Several studies have been conducted within the sub-Saharan region on the molecular epidemiology of *Cryptococcus neoformans*, but limited data are available from Zimbabwe (Beale *et al.*, 2015; Kangogo *et al.*, 2015; Litvintseva *et al.*, 2005; Van Wyk *et al.*, 2014). This is the first report to our knowledge to describe the population diversity of *Cryptococcus neoformans sensu stricto* among a cohort of HIV-associated CM patients living in Zimbabwe.

The median age of the cohort was 36 years with an age range of 18 to 58 years, and this is similar to that found in previous studies conducted in Zimbabwe (Hakim *et al.*, 2000; Heyderman *et al.*, 1998; Makadzange *et al.*, 2010). Worldwide, it has generally been observed that CM mainly affects people between the ages of 20 and 50 years (Day, 2004). In this study, it was also observed that there were more males (57 %) affected compared to females, and similar findings were observed in other major studies conducted in Zimbabwe (Hakim *et al.*, 2000; Heyderman *et al.*, 1998; Jarvis *et al.*, 2010; Makadzange *et al.*, 2010). The difference in the infection rates between males and females is thought to be influenced by the gender-specific host immune response, although this phenomenon is not well studied (McClelland *et al.*, 2013).

Fifty-five (83.3 %) *Cryptococcus neoformans* clinical isolates were available for molecular epidemiological investigations, and the majority of the isolates were of genotype AFLP1/VNI (72.7 %), followed by genotype AFLP1A/VNB/VNII with a prevalence of 14.6 %. The lowest number of isolates from this cohort of patients was *Cryptococcus neoformans* genotype AFLP1B/VNII (12.7 %). These observations were consistent with those from studies conducted in South Africa

(Beale *et al.*, 2015; Van Wyk *et al.*, 2014). *Cryptococcus neoformans* AFLP1/VNI genotype has a global distribution, contrary to the genotypes AFLP1B/VNII and AFLP1A/VNB/VNII that tend to be restricted to the African continent (Cogliati, 2013). Little is known about the clinical course of infections caused by *Cryptococcus neoformans* genotypes AFLP1B/VNII and AFLP1A/VNB/VNII. The majority of the isolates were mating-type α (n=51; 92.7 %), and only four (7.3 %) were mating-type **a**. The second highest African prevalence of mating-type **a** was found among the Zimbabwean isolates, and this was consistent with findings from Botswana (n=14/139; 10.1 %) (Litvintseva *et al.*, 2003). The high prevalence of mating-type **a** in the African population suggests the possibility of recombination of these genotypes within the environment. Surprisingly, the linkage disequilibrium tests (I_A and $\hat{r}_d$) were observed to be highly significant, which indicates that it is less likely that recombination is playing a role in the studied *Cryptococcus neoformans sensu stricto* population (Table 3). However, the Simpson's diversity index shows that there is high genetic diversity, which is in contradiction with the outcome of the I_A and $\hat{r}_d$ tests. A similar observation was recently described in a population study that included isolates from Botswana; both clonality and recombination were observed with different approaches (Chen *et al.*, 2015).

The majority of patients (n=54/55; 98.2 %) infected with *Cryptococcus neoformans sensu stricto* presented with severe headache having a median duration lasting 14 days, and this was consistent with other studies (Heyderman *et al.*, 1998; Makadzange *et al.*, 2010; McCarthy *et al.*, 2006). It was not possible to determine viral loads which would have given a broader assessment of immunological suppression. The $CD4^+$ count of HAART-experienced and -naïve patients was similar when compared statistically (P=0.78). There was also no difference observed in the $CD4^+$ counts of patients infected with the different genotypes of *Cryptococcus neoformans sensu stricto* (P=0.79).

The patients had a median hospital stay (interquartile range) of 16 (11–23) days that was consistent with findings from other cohorts and observational studies from South Africa and Zambia (Mwaba *et al.*, 2001; Sogbanmu *et al.*, 2014). This usually reflects complications in the management of CM patients. An overall in-hospital mortality of 55.6 % was observed among the HIV-associated CM co-infected patients in this study. The in-hospital mortality was high when compared with other studies done locally and those performed within the region that recorded mortality rates between 27.0 % and 47.0 % (Beale *et al.*, 2015; Beardsley *et al.*, 2016; Hakim *et al.*, 2000; Kambugu *et al.*, 2008; McCarthy *et al.*, 2006). Most likely, the mortality rate in this study could have risen if the patients were followed up for a longer period of time as outpatients.

In this study, we did not observe an association between the different genotypes of the Zimbabwean *Cryptococcus neoformans* isolates and patient clinical outcome, as was demonstrated in other studies done in sub-Saharan Africa (Wiesner *et al.*, 2012; Beale *et al.*, 2015). However, despite the small group of patients available for analysis, the length of hospital stay and being on HAART were statistically significantly associated with the patient clinical outcome. Patients on HAART were more likely to die compared to those not on HAART. These findings demonstrate that mortality as a result of HIV-associated CM is still a major problem in Zimbabwe, despite the availability of antiretroviral and antifungal therapy.

A nine-marker microsatellite typing panel was used to further sub-genotype the *Cryptococcus neoformans* isolates from Zimbabwe to infer comparisons to other parts of Africa. Microsatellite typing is a highly discriminatory typing approach, and it allows *Cryptococcus neoformans* isolates from different origins to be distinguished (Illnait-Zaragozi *et al.*, 2010). Our findings demonstrate that some genotypes of *Cryptococcus neoformans* were more closely related to each other than to other genotypes. Interestingly, we also found a high genetic diversity among the Zimbabwean isolates themselves, as well as when they were compared with *Cryptococcus neoformans* isolates collected from different parts of Africa (Bogaerts *et al.*, 1999; Swinne *et al.*, 1986, 1989, 1991). We were unable to find a link between MCs and *Cryptococcus neoformans sensu stricto* AFLP genotypes; this might be due to presence of recombination within and among genotypes AFLP1/VNI, AFLP1A/VNB/VNII and AFLP1B/VNII. A similar observation was made in a Dutch set of 245 clinical *Cryptococcus neoformans sensu stricto* isolates, although only seven isolates were genotype AFLP1B/VNII; no significant correlation was found despite that these isolates were grouped together (Hagen *et al.*, 2012).

In summary, this study presents the first molecular epidemiological survey in Africa to compare the genotypic diversity of *Cryptococcus neoformans sensu stricto* from clinical, environment and veterinary samples. We also present data on the prevalence of *Cryptococcus neoformans sensu stricto* genotypes and their mating types among HIV-infected subjects in Zimbabwe. It appears that Zimbabwean *Cryptococcus neoformans sensu stricto* clinical genotypes are highly polymorphic, and the presence of both mating types **a** and α suggests a possibility of recombination of these genotypes within the patient and/or environment.

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