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Clinical epidemiology and high genetic diversity amongst Cryptococcus spp. isolates infecting people living with HIV in Kinshasa, Democratic Republic of Congo

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Clinical epidemiology and high genetic diversity amongst *Cryptococcus* spp. isolates infecting people living with HIV in Kinshasa, Democratic Republic of Congo

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Abstract

Neuromeningeal cryptococcosis (NMC) due to *Cryptococcus* spp. complex is one of the life-threatening opportunistic infections during HIV infection, mainly in sub-Saharan Africa. We focused on the molecular characterization of *Cryptococcus* isolates from people living with HIV (PLHIV) in Kinshasa (DRC) and investigated possible associations between NMC severity factors and the *Cryptococcus neoformans (Cn)* multilocus sequence typing (MLST) profiles. The isolates were characterized using PCR serotyping, MALDI-TOF MS, internal transcribed spacer (ITS) sequencing and MLST analysis. NMC severity factors, such as hypoglycorrhachia (<50mg/dL), very raised cerebral spinal fluid opening pressure (>30 cm water), and pejorative outcome in patients were compared with the *Cn* MLST sequences type (ST). Twenty-three out of 29 *Cryptococcus* isolates have been identified as serotype A using PCR (79.3%; 95% IC: 65.5-93.1), while six (20.7%; 95% IC: 6.9-34.5) were not serotypable. The 29 isolates have been identified by ITS sequencing as follows: *Cryptococcus neoformans* (23/29, 79.3%), *Cryptococcus curvatus* (5/29, 17.2%), and *Cryptococcus laurentii* (1/29, 3.5%). All *Cn* isolates were identified as molecular type VNI using the MLST ISHAM scheme, including seven different STs: ST93 (n=15), ST5 (n=2), ST53 (n=1), ST31 (n=1), ST4 (n=1), ST69 (n=1), and one novel ST identified in the present work and subsequently assigned as ST659 (n=2). Among NMC severity factors, only the patient pejorative outcome was associated with infections by less common STs isolates (7/8, 87.5%, p=0.02) (ST53, ST31, ST5, ST4, ST659, and ST69). Molecular analysis of *Cryptococcus* spp. isolates showed a wide species diversity and genetic heterogenicity of *Cn* within the VNI molecular type. Furthermore, infections due to less common STs were associated with more pejorative comes than those due to ST93.

Keywords

Cryptococcus spp., species diversity, ITS sequencing, MALDI-TOF MS, Multilocus sequence typing, people living with HIV, neuromeningeal cryptococcosis, Kinshasa, DRC

INTRODUCTION

Among opportunistic infections encountered during HIV/AIDS, neuromeningeal cryptococcosis (NMC) is implied in 15% of deaths and 75% of which occur in sub-Saharan Africa [1]. In this region, the annual mortality from this invasive fungal infection is estimated at 504,000 per year, making it the fourth leading cause of death from infectious diseases [2].

Among 510,000 people living with HIV (PLHIV) in the Democratic Republic of Congo (DRC), only 75% are on antiretroviral treatment (ART) and the NMC prevalence is estimated at 8.8%, with a death rate of approximately one out of three patients [3], [4].

Grounded on epidemiological, pathobiology, geographical distribution, ecological niches, clinical presentation, therapeutic, and genetic differences [5], the *Cryptococcus neoformans/C. gattii* species complex (*Cn/Cg*), the main etiological agents of cryptococcosis, are classified into two species, four varieties, and eight major molecular types [6]. In this manuscript, we applied the new nomenclature of the *Cryptococcus neoformans/C. gattii* species complex as proposed by Ferry Hagen *et al*. [7].

Apart from the *Cn/Cg* species complex, non-*neoformans*/ *gattii Cryptococcus* species which have long been considered saprophytic and non-pathogenic to humans have recently been associated with cryptococcal infections. Of these species, *C. laurentii* and *C. albidus* are identified in 80% of the cases [8]. Some *Cryptococcus* spp., such as *C. gattii* require a more intensive therapeutic approach to management than *C. neoformans*, and others, such as non-*neoformans* and non-*gattii* are known to have primary resistance to fluconazole and 5-flucytosine [9]. Hence, local epidemiological knowledge including circulating *Cryptococcus* spp. molecular types and their susceptibility profiles to usual antifungal agents would facilitate better cryptococcosis surveillance in the general population, and update patients' management based on local data.

As the association of *Cn/Cg* molecular type with the antifungal susceptibility profile has previously been established, it is also opportune to verify the association of molecular types (MT) or even MLST sequence types (ST) with the cryptococcosis clinical presentation. Therefore, we hypothesized that the NMC severity factors could be associated with the isolates ST in the cause of the disease [10].

Therefore, we describe here the NMC clinical epidemiology amongst PLHIV and the molecular characterization of *Cryptococcus* spp. isolates. In addition, the association between NMC severity factors and *Cryptococcus neoformans* MLST ST was statistically analysed.

MATERIALS AND METHODS

Study design, patients and samples

A cross-sectional study was conducted in three Kinshasa public hospitals supported by Doctors without Borders-Belgium (MSF), from 1 February 2019 to 29 February 2020. Thus, 278 patients were included and among them, NMC was diagnosed based on the cryptococcal antigens (CrAg) detection among patients and/or the presence of yeasts cells detected by India ink staining and/or by culture.

Biological analyses

The CrAg detection was carried out in the CSF of each included patient, using the CrAg LFA IMMY test (Immuno-mycologic, Norman, OK, USA). Direct staining with India ink in the CSF was also carried out, and the CSF was cultured on Sabouraud Dextrose Agar-Chloramphenicol medium (SDA-C, bioMérieux, France) at 30°C for 48 to 72h. The qualitative test Pandy was performed for determining proteinorachia as previously described [11].

Identification by MALDI-TOF MS

MALDI-TOF MS (Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry, Bruker Daltonics GmbH, Germany) was used for the identification of all fungal strains. From the culture on SDA-C, an extended direct deposit was performed by adding 1µL of 70% formic acid to the sample on a MALDI target plate (MSP 96 BC ground steel target; Bruker Daltonics). Then, 1µL of saturated cyano-4-hydroxycinnamic acid solution (HCCA matrix; Bruker Daltonics) was added. Each microorganism tested was spotted twice on the same MALDI target plate. Measurement was performed with MALDI Flex control V3.4 (Bruker Daltonics) following the settings suggested by the manufacturer using automated collecting spectra. The spectra of each duplicated spot were compared with those in the reference library (BD 8326 or version V 9.0) [12]. The following score was considered for the identification of the fungal species: MS Score $\geq$ 1.5 and the 3 first results identical and consistent with the appearance of the colonies on agar.

Molecular analysis

DNA extraction

Genomic DNA was extracted from the fresh 24-hour cultures using the NucleoSpin blood quick pure kit (Macherey-Nagel, Düren, Germany). Two preliminary steps were added to the manufacturer's protocol, namely bead-beating and thermal shock. In a 2mL tube containing 0.5mm glass beads (Roche Diagnostics GmbH, Penzberg, Germany), colonies were mixed with 350µL lysis buffer (Promega Corporation, USA). The mixture was vortexed five times due to 6000 vibrations per minute for 40 seconds (bead-beating). Between each pass, the tube was cooled between -20°C and 1°C for 30 seconds in a Nalgene microtube cooler container (Dutscher, France) for a thermal shock.

Serotyping PCR

A classical serotyping PCR designed for *Cn/Cg* species complex was performed according to the protocol described by Ito-Kuwa *et al*.[13].

ITS sequencing

The ITS2 region of the rRNA gene cluster was amplified using the ITS86 forward primer 5′GTGAATCATCGAATCTTTGAA 3′ and ITS4 reverse primer 5′TCCTCCGCTTATTGATATGC 3′ [14]. The amplified products were purified using the kit clean Seq Agencourt (Beckman Coulter Life Science). The sequencing was done on the automate ABI 3500/3500XL (Applied Biosystem, Life Technologies). Bidirectional sequence data were generated after purification using the BigDye terminator sequencing kit (Applied Biosystems, Life Technologies, Belgium). Sequences generated by the software ABI Sequence Scanner V.1.0 (Applied Biosystems, Life Technologies) were then compared to the CBS database by using The BioloMICS database software (https://wi.knaw.nl/page/Pairwise_alignment), which comprises several databases including Genbank. Only results that repeated the same identification at least three times and had a similarity score greater than 95% were considered valid.

Multilocus sequence typing

Multilocus sequence typing (MLST) was performed using the International Society of Human and Animal Mycology (ISHAM) consensus scheme for the *Cn/Cg* species complexes; including six unlinked housekeeping loci (GPD1, LAC1, URA5, SOD1, CAP59, and PLB1) and the non-coding region IGS1 [6]. After DNA extraction, samples were sequenced using Illumina HiSeq as previously described [15], and the raw contigs sequences were paired, removed of duplicate reads, and trimmed using Geneious Prime 64_2021_1 (https://www.geneious.com). Then, the MLST loci were extracted by mapping to the reference sequences of each MLST locus from the online ISHAM MLST fungal database (https://mlst.mycologylab.org/) and each allele type (AT) was assigned using the same online database. The ATs combination defined the sequences type (ST) which in most cases corresponds to the species Molecular type.

Phylogenetic analysis

Phylogenetic analysis of concatenated sequences of the seven MLST loci was performed using MEGA v.6.06 software (http://www.ebi.ac.uk/tools/msa/clustalo). A dendrogram was produced by the Maximum Likelihood method using sequences alignment with the Kimura 2-parameter method. Gaps were treated as a complete deletion. Statistical support for each clade was assessed using bootstrap analysis with 1000 replicates. Apart from *C. neoformans* and *C. gattii* reference strains (WM) included in the analysis, the MLST sequences of the only *C. neoformans* strain (ZS) previously isolated from Congolese infected patient (DRC) was also included.

Statistical analysis

The analysis was carried out using R-cmdr version 2.6-1 (R Foundation for Statistical Computing, Vienna, Austria). Missing data were considered completely random and the available data were analyzed. The continuous variables were summarised as mean ± standard deviation and compared using Student's t-test. The proportions and their respective 95% confidence intervals were calculated for the categorical data. The main outcome variable was the NMC diagnosis. This variable was compared to other variables of the same category using Pearson's chi-square test or Fisher's exact test if the expected values were less than five. Very raised CSF opening pressure (>30cm water), hypoglycorrhachia (<50mg/dL) and patients' pejorative outcome were considered in performing the association analysis between the NMC severity factors and the identified ST-MLST profile. Also, two isolates categories were formed according to the STs-MLST profile: the main ST isolated on the one hand, and the other STs on the other hand. All tests were two-tailed and a $p < 0.05$ was considered statistically significant.

It is noteworthy that comparative data between PLHIV with *Cryptococcus neoformans* versus *Cryptococcus curvatus*/ *C. laurentii* meningitis are presented in another published paper in BMC infectious diseases (https://doi.org/10.1186/s12879-021-06849-3).

Ethical considerations

This work was carried out in strict compliance with ethical rules, with the approval of the Ethics Committee of the Public Health School of the Faculty of Medicine of the University of Kinshasa under the approval number ESP/CE/071/2019. All patients included in this study were informed of the risks associated with the study and gave their informed consent to participate. Anonymity was guaranteed and the data collected was kept and handled by the research team alone.

RESULTS

Among 278 PLHIV included, 66 (23.7%, 95% CI: 18.7 – 28.8) had NMC. However, the NMC prevalence was almost similar in men (24.8%, 95% CI: 14.9 – 34.7, 25/101 included men patients) as in women patients (23.2%, 95% CI: 15.7 – 30.7, 41/177 included women patients).

Patients' characteristics

The demographic and clinical characteristics of the patients are presented in Table 1. The mean age of the included patients was 42.2±12.1 years old. Most of them were female (63.7%), married or cohabitating (49.3%), and had a secondary education level (55·8%). Regarding the patients' clinical stage before NMC diagnosis, NMC tends to develop during HIV-infection stage IV (95.5%, $p = 0.0008$). PLHIV with NMC have four times more probability to present headaches (OR 3.8; IC 95%: 1.7 – 8.8; p=0.0001), three times more probability to present convulsions (OR 2.7; IC 95%: 1.01-7.1, p=0.02); and six times more probability to present visual disturbances than no NMC patients (OR 5.6; IC 95%: 1.04-37.5, p=0.02). The vast majority of NMC patients had clear CSF (93.9%) and a significantly raised CSF

200 opening pressure (65.4%, p<0.0001). Furthermore, the pejorative outcome was not significantly
201 different between NMC than non-NMC patients (37.5 versus 35.4%, respectively).

202 Table 1: Demographic and clinical characteristics of patients

Characteristics[1]	Overall data (%)[2]	NMC		*p*-value	Crude OR (95% CI)
		No (%)	Yes (%)		
Demographic characteristics					
Mean age ± SD (years) (n=278)	42.2 ± 12.1	42.8 ± 12.0	40.2 ± 12.4	0.1	
Female sex (n=278)	177 (63.67)	136 (64.2)	42 (62.1)	0.7	
Marital status (n=278)				0.6	
Single	89 (32.01)	71 (33.5)	18 (27.3)		
Married/cohabitating	137 (49.28)	102 (48.1)	35 (53.0)		
Divorced/widower	52 (18.7)	39 (18.4)	13 (19.7)		
Education level attained (n=278)				0.6	
None/primary	69 (24.82)	52 (24.5)	17 (25.8)		
Secondary	155 (55.76)	121 (57.1)	34 (51.5)		
Higher education/university	54 (19.42)	39 (18.4)	15 (22.7)		
Clinical characteristics					
HIV clinical stage (n=242)				0.0008	-
Stage I	1 (0.41)	1 (0.5)	0 (0.0)		
Stage II	2 (0.83)	2 (0.9)	0 (0.0)		
Stage III	51 (21.07)	48 (22.6)	3 (4.5)		
Stage IV	224 (92.6)	161 (75.9)	63 (95.5)		
Headaches (n=269)	174 (64.68)	120 (58.8)	54 (84.4)	0.0001	3.8 (1.7-8.8)
Fever (°C) (n=269)	179 (66.54)	141 (68.8)	38 (59.4)	0.1	
Weight loss (n=269)	144 (53.53)	107 (52.2)	37 (57.8)	0.4	
Consciousness disorder (n=269)	100 (37.17)	82 (40.0)	18 (28.1)	0.08	
Memory impairment (n=268)	74 (27.61)	59 (28.9)	15 (23.4)	0.3	
Neck stiffness (n=269)	49 (18.22)	33 (15.6)	16 (24.2)	0.1	
Vomiting (n=269)	54 (20.07)	42 (20.5)	12 (18.8)	0.7	
Convulsions (n=269)	23 (8.6)	13 (6.3)	10 (15.6)	0.02	2.7 (1.01-7.1)
Vertigo (n=269)	32 (11.9)	23 (11.2)	9 (14.1)	0.5	
Brudzinski sign (n=269)	15 (5.58)	8 (3.8)	7 (10.6)	0.05	
Physical asthenia (n=269)	30 (11.1)	23 (11.2)	7 (10.9)	0.9	
Kernig sign (n=269)	14 (5.2)	9 (4.2)	5 (7.6)	0.3	
Visual disturbances (n=269)	8 (2.97)	3 (1.5)	5 (7.8)	0.02	5.6 (1.04-37.5)
Functional Impotence (n=269)	17 (6.32)	12 (5.9)	5 (7.8)	0.5	
Clear CSF appearance (n=265)	249 (93.9)	200 (94.3)	62 (93.9)	1	
Very raised CSF opening pressure (cm of water) (n=92)	65 (70.6)	7 (10.6)	17 (65.4)	<0.0001	-
Antiretroviral therapy (ART) (n=278)	204 (73.4)	153 (72.2)	51 (77.3)	0.4	-
Pejorative outcome[3] (n=217)	78 (35.9)	57 (35.4)	21 (37.5)	0.7	-

[1]according to available data

[2]column per cent calculated for each group

[3]Death, status quo, discharge against medical advice, or transfer due to complications

Routine diagnostic analysis of NMC

Out of 66 NMC samples confirmed, 63 (95.5%, 95% CI: 89.4-100) had detectable cryptococcal antigen, only 29 (43.3%, 95% CI: 31.8–56.1) had yeasts present after by India ink staining, and the repeated culture was positive only in 43.3% of the cases (29/66). All three CrAg negative samples were recovered from positive cultures.

MALDI-TOF MS, ITS sequencing, and PCR serotyping characterization

Of the 29 positive cultures, MALDI-TOF MS identified 23 as *C. neoformans* (79.3%), four as *C. curvatus* (13.8%), and two (6.9%) could not be identified. While only 23 isolates were identified as serotype A using serotyping PCR (79.3%), ITS sequencing identified all isolates as *C. neoformans* 79.3% (23/29), *C. curvatus* 17.2% (5/29), and *C. laurentii* 3.5% (1/29). The results of the MALDI-TOF MS, ITS sequencing, and serotyping PCR characterization are summarized in Table 2.

Table 2: MALDI-TOF MS, ITS sequencing, and Multiplex PCR serotyping characterization

Analysis	n=29 (%)
MALDI-TOF MS	
Cryptococcus neoformans	23 (79.3)
Cryptococcus curvatus	4 (13.8)
Not identified	2 (6.9)
ITS sequencing	
Cryptococcus neoformans	23 (79.3)
Cryptococcus curvatus	5 (17.2)
Cryptococcus laurentii	1 (3.5)
Serotyping PCR	
Serotype A	23 (79.3)
No identifiable	6 (20.7)

MLST result

Apart from the six strains identified as *C. curvatus or C. laurentii*, the remaining 23 *C. neoformans* isolates belong to the molecular type VNI. MLST analysis identified seven different STs: ST93 (15 isolates, 65.2%), ST5 (two isolates, 8.6%), ST53 (one isolate, 4.3%), ST31 (one isolate, 4.3%), ST4 (one isolate, 4.3%), ST69 (one isolate, 4.3%), and one novel ST that was not yet reported in the online fungal MLST database and was later assigned as ST659 (two isolates, 8.6%).

Phylogenetic analysis

Phylogenetic analysis using maximum likelihood identified two major clusters among the studied isolates investigated (BZ isolates in figure 1), grouping ST659, ST69, ST4, ST5, and ST93 including 21 isolates out of 23 analysed. The two remaining STs (ST31 and ST53) were slightly less correlated with the other cluster. The single Congolese (DRC) isolate previously characterised and stored in the MLST fungal database was deeply embedded in the first cluster (Fig1).

Fig1. Phylogenetic tree based on concatenated sequences of the seven MLST loci: CAP59, GPD1, IGS1, LAC1, PLB1, SOD1, and URA5 using maximum likelihood. Numbers near the nodes represent the bootstrap values obtained by 1000 repetitions.

NMC severity factors and MLST ST of *Cryptococcus neoformans* isolates

Among NMC severity factors described and considered in the present study, only the pejorative therapeutic outcome was associated with infections due to the less common MLST STs isolates (ST5, ST659, ST53, ST31, ST4, and ST69) versus the main ST (ST93) (87.5% vs. 40%, respectively; p=0.02). Table 3 summarizes the NMC severity factors compared to the MLST ST of the *C. neoformans* isolates.

Table 3: NMC severity factors compared to the MLST STs of the *C. neoformans* isolates.

Variable	*Cryptococcus neoformans* ST identified		p
	Main study ST[1] n[3] (%)[4]	Less common STs[2] n[3] (%)[4]	
Glycorhachia (mg/dl) (n=10)			1
Low (≤ 50)	7 (87.5)	2 (100)	
High (≥ 60)	1 (12.5)	0	
Opening pressure (cm of water) (n=8)			0.4
Moderately high (<30)	2 (40)	0	
Very high (≥30)	3 (60)	3 (100)	
Therapeutic outcome (n=23)			0.02
Good[5]	9 (60)	1 (12.5)	
Bad[6]	6 (40)	7 (87.5)	

[1]ST93

[2]STs5, 659, 53, 31, 4, 69.

[3]With available data

[4]Percentage of columns calculated for each group

[5]Recovery and discharge from hospital

[6]Death, status quo, discharge against medical advice, or transfer due to complications

DISCUSSION

We described the clinical epidemiology of PLHIV, the routine analysis and the molecular characterization of the *Cryptococcus* spp. isolates. In addition, the association between the NMC severity factors and *Cryptococcus neoformans* MLST ST was statistically tested.

Neuromeningeal cryptococcosis (NMC) prevalence in people living with HIV (PLHIV) was estimated at 23.7% (95% CI: 18.7 – 28.8). This hospital prevalence is much higher than that reported in France [16], in other Africa states [17], [18], and previously in the DRC [4]. This could be explained by (a) the difference in HIV infection prevalence in various countries and regions, (b) the HIV management and the opportunistic infections prevention policy applied in each country, (c) the HIV patients focused on in each study, and (d) the sensitivity and specificity of biological analysis used for diagnostic confirmation. Reflecting the PLHIV demographic profile in the DRC, female patients aged 42.19 ± 12.13 years old, married/cohabitating and with secondary education level were non-significantly most affected. McClelland E. *et al*. found that virulent *C. neoformans* phenotypes from females had longer doubling times and released more capsular glucuronoxylomannan (GXM) in the 17-β estradiol presence. Plus, macrophages from women phagocytized more *C. neoformans* than those from men. The men hence had a higher fungal load than women and their macrophages were more likely to be destroyed by *C. neoformans* [19]. This protective trend in women was not significantly noted in the current study. Headache, convulsions and visual disturbances were significantly associated with NMC. This data is largely consistent with the literature [20]. Slightly more than the proportion reported in this study (7.7%), visual disturbances are known to be associated with NMC in 18% of cases following raised intracranial pressure [21]. Among the clinical parameters (headache, sensorium depression, papilledema, and raised CSF opening pressure) and the radiological ones (flattening of the posterior sclera, increased CSF in the subarachnoid space around the optic nerve, optic nerve tortuosity and empty parietal saddle) defining these disorders, only headaches and raised CSF opening pressure were found in the present study [21]. NMC was associated with higher raised CSF opening pressure, CD_4 count < 100 cells/mm^3 and HIV-infection stage IV. One of the most critical outcome determinants in PLHIV with NMC is the raised opening CSF pressure which is generally correlated with high CSF fungal load, morbidities and increased risk of death [22]. In agreement with the data described by Bicanic *et al*, 63% of patients in the present study had very high opening CSF pressure [23].

Cryptococcal antigen detection was the main diagnostic tool for NMC in the present study (95% positivity rate out of 66 confirmed samples). Compared to other studies, culture positivity rate, and *Cryptococcus* India ink staining identification were very low in the present study [24]. For Kabanda T. *et al*, CrAg detection has shown more sensitivity in clinical situations (100%) than culture (95.7%) and India ink (93.6%) [25]. The low positivity rate of direct microscopy and culture found in the present study could be caused by the precarious storage conditions of samples before analyses and/or probable low CSF fungal load in certain samples [26]. The ITS sequencing gave a better isolates identification (29/29). Described as the main species responsible for cryptococcosis diseases in PLHIV [27], *C.*

neoformans was identified for 73.3% of all isolates. The remaining cases were identified as *C. curvatus* (17.2%) and *C. laurentii* (3.5%). Initially considered saprophytic and non-pathogenic to humans, the non-*neoformans* and non-*gattii Cryptococcus* species are increasingly found in clinical infections in recent years [8]. Although the *C. neoformans* NMC clinical presentation is more severe in PLHIV than in non-*neoformans*/ non-*gattii* NMC, *C. curvatus* and *C. laurentii* have a high tendency to be fluconazole and 5-flucytosine resistant. In addition, these two latter species are more difficult to identify by routine laboratory methods than *C. neoformans* [8]. Thus, out of six non-*neoformans* and non-*gattii* isolates, only four were correctly identified by MALDI-TOF MS and confirmed by ITS sequencing. The lack of certain species reference spectra in the database provided by the mass spectrometry manufacturer (Bruker: BD 83) for identification, and genome differences between *Cryptococcus* species, could explain these results. Worldwide, serotype A isolates remains the most commonly isolated in environmental and clinical settings [28], a trend also observed in the current study.

The MLST analysis of *C. neoformans* isolates revealed large heterogeneity of STs within the single molecular type found in the present study (VNI), involving seven distinct STs. In line with our results, *Cryptococcus neoformans* ST93 is the most isolated in various countries (China, India, Indonesia, South Africa, Thailand, Brazil, Uganda, and Colombia), both in clinical and environmental settings. It has been associated with high mortality in Uganda [29]. In this study, ST93 was associated with a less pejorative treatment outcome than the less common STs. It hence opens up the debate on the relative virulence of each ST compared to the others. Although most of the STs had already been isolated in the DRC neighbouring countries, namely ST4 in Uganda and Tanzania, ST5, ST31, ST69, and ST93 in Uganda only, one ST (ST53) had previously been isolated only in Thailand and in no other country worldwide (https://mlst.mycologylab.org/). Plus, one isolate had an ST identified for the first time in the present study, subsequently assigned as ST659. The Congolese strain (ZS CN ST32) isolated 30 years earlier is closely related to the isolates of the dominant ST (ST93) identified in the current study.

As described by Trilles *et al.* regarding the low antifungal susceptibility of VGI isolates compared to the other molecular types tested [30], the less common STs isolates identified in the current study were associated with poor therapeutic outcomes.

CONCLUSIONS

A more severe epidemiological profile of NMC than previously reported from the DRC was found using a panel of diagnostic tests in symptomatic PLHIV. Apart from the species diversity identified amongst the yeasts isolated from CSF samples, including *C. neoformans*, *C. curvatus* and *C. laurentii*, the STs within the single molecular type VNI identified in the present study showed great heterogeneity, including seven different STs with one major ST (ST93) and six less common STs (ST5, ST659, ST53, ST31, ST4, and ST69). In addition, the less common STs isolates were associated with the patient

pejorative outcomes. More robust studies including larger sampling numbers and antifungal susceptibility of isolates could improve the understanding of the data from this study.

AUTHOR CONTRIBUTIONS

Conceptualization: BZB.

Clinical and biological analysis: BZB, RS, RB and AK.

Samples and patient clinical data collection: GM, NL and PRM.

Data curation: BZB.

Funding acquisition: GML and MPH.

Investigation: BZB.

Methodology: BZB.

Project administration: MPH.

Resources: BZB and RS.

Supervision: MPH.

Validation: BZB, GM, NL, PRM, RS, RB, AK, HSN, PKZ, MMM, CNM, WM, GML and MPH.

Visualization: BZB.

Writing – original draft: BZB.

Writing – review & editing: HSN, PKZ, MMM, CNM, MM, WM, GML, and MPH.

TRANSPARENCY DECLARATION

The authors declare no conflict of interest either in the conduct of the study or in the publication of this report.

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Figure

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