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Screening for cryptococcal antigenemia and meningeal cryptococcosis, genetic characterization of *Cryptococcus neoformans* in asymptomatic patients with advanced HIV disease in Kinshasa, Democratic Republic of Congo

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We evaluated the prevalence of serum and meningeal cryptococcosis in asymptomatic outpatients with advanced HIV disease (CD4 < 200 cells/mm3) in a cross-sectional screening context in Kinshasa clinics (DRC). Lumbar puncture (LP) was performed in patients with positive serum cryptococcal antigen (CrAg) test, and *Cryptococcus* spp. isolated from cerebrospinal fluid (CSF) were identified by MALDI-TOF-MS, and characterized using serotyping-PCR, ITS-sequencing and multilocus sequence typing (MLST). The genetic profiles obtained were then compared with those of isolates previously described in symptomatic patients in the same clinics. Forty-seven patients with advanced HIV disease out of 262 included were positive for serum CrAg (18%, 95% CI: 14.2–24.3). The prevalence of asymptomatic cryptococcal meningitis (CM) was then measured at 50% among patients with positive serum CrAg test who consented to LP (19/38). Only four CSF samples were culture positive and all were characterized as *Cryptococcus neoformans*, molecular type VNI and belonging to two different sequence types (ST): ST93 (3/4) and ST63 (1/4). While ST93 is also the main genomic profile described in advanced HIV disease patients with symptomatic CM in Kinshasa clinics, ST63 has not yet been identified in DRC before. It is likely that future studies involving a large number of strains will be necessary before any definitive conclusions can be drawn on the involved strains in asymptomatic patients.

Keywords Cryptococcal antigen screening, People living with HIV, Cryptococcosis, Molecular characterization, Antifungal susceptibility testing, DRC

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Cryptococcal meningitis (CM) is a deep-seated mycosis induced by yeast species belonging to the *Cryptococcus* genus, of which seven species are mainly known to be pathogenic: *Cryptococcus neoformans sensu stricto* (*s.s.*), *C. deneoformans*, *C. gattii s.s.*, *C. bacillisporus*, *C. decagattii*, *C. deuterogattii*, *C. tetragattii*; and three non-pathogenic: *C. amylolentus*, *C. depauparatus* and *C. luteus*[1–3]. CM is responsible for approximately 19% (about 112,100 subjects) of deaths in advanced HIV disease patients each year worldwide, mainly in sub-Saharan Africa[4].

In the HIV-positive population, efforts to end CM deaths are currently focused on active screening of serum and then cerebrospinal fluid (CSF) for cryptococcal antigen (CrAg) in patient with new HIV diagnosis, returning to care or failing antiretroviral treatment (ART) developing advanced HIV disease (CD4 < 200 cells/mm3 or a WHO clinical stage 3 or 4 event), followed by pre-emptive antifungal treatment for positive patients, combined with ART 4 to 6 weeks after the start of antifungal therapy[5,6].

Approximately 96,211 people living with HIV (PLHIV) in the Democratic Republic of Congo (DRC) have been identified as being at high risk of developing CM in the context of advanced HIV disease due to a lack of ART and treatment failure for those who do receive it. Thus, in 2020, an estimated 4,883 PLHIV would have died of cryptococcosis[7]. Despite this heavy burden, no policy to prevent cryptococcal infections is effectively integrated into the DRC national HIV/AIDS program.

In routine medical practice, two circumstances lead to the diagnosis of CM in the Kinshasa clinics supported by Médecins sans Frontières (MSF-Belgium), namely (1) PLHIV with overt, though not very expressive, symptoms suggestive of CM, with positive diagnostic tests; (2) asymptomatic PLHIV with serum and then CSF positive diagnostic tests during the systematic screening recommended for any patient with advanced HIV disease. Considering that some cryptococcal species such as *C. bacillisporus* and *C. deuterogattii* preferentially affect immunocompetent patients, and that *C. neoformans*, *C. gattii s.s.*, *C. tetragattii* and *C. decagattii* are selectively incriminated in HIV immunocompromised patients[1,3,8], the aforementioned difference in clinical presentation here could be associated with a difference in species profile or sequence type within the molecular types of *Cryptococcus* spp. involved in each patient category considered in this study. Thus, we wondered whether patients with asymptomatic CM were infected with *Cryptococcus* isolates with a different genetic profile than those infecting symptomatic patients in the Kinshasa clinics[9]. In order to further assess the characteristics of the strains likely to induce insidious disease in these vulnerable patients, we thought it advisable to also test their susceptibility to the usual antifungal agents.

In this study, we therefore sought to assess the prevalence of cryptococcal antigenemia and asymptomatic CM in outpatients with advanced HIV disease, in a screening setting. Then, for proven cases of asymptomatic CM, we characterized the *Cryptococcus* isolates involved and determined their in vitro susceptibility to the main antifungals.

Ultimately, the data on cryptococcosis screening described in this HIV-infected population will form a basis on which the Congolese national HIV/AIDS program could build a country-wide screening system.

Results

High prevalence of cryptococcal antigenemia and cryptococcal meningitis in outpatients with advanced HIV disease

During 14 months of study, 262 asymptomatic outpatients with advanced HIV disease (CD4 < 200/µL) were screened for serum CrAg. Of those, 47 (17.9%, 95% CI: 14.2–24.3) were positive. In the overall study population, patients with CD4 counts below 100 cells/µL were as likely to have serum CrAg as those with CD4 counts between 100 and 200 cells/µL (69.6% versus 30.4%, OR 0.5; 95% IC: 0.2–1.0, $p = 0.03$). Considering the 43 sera tested for antigenic titer (four sera lost due to inadequate storage), approximately two-thirds of patients [27/43, (62.8%, 95% CI: 47.6–78.0)] had a high antigenic titer (≥ 1/160). Among patients with positive serum CrAg, 80.8% (38/47) consented to lumbar puncture according to the proposed protocol, and 19 out of 38 (50%, 95% CI: 33.8–66.2) were CSF CrAg positive. This represents 7.2% of all patients with advanced HIV disease who were initially screened (Fig. 1). In addition, a positive CrAg test in the CSF was significantly associated with a high serum CrAg titer (73.7%, 14/19 versus 26.3%, 5/19, $p = 0.01$, ≥ 1/160 and < 1/160 respectively).

Demographic and clinical characteristics of patients

The study participants came from the four constituent districts of Kinshasa, with no significant predominance ($p = 0.5$). The mean age of included patients was 40.7 ± 13.2 years old. According to the available cross-referenced data, female population was the most numerically represented in this study (64%, 161/251). The rate of CrAg positivity in serum was almost similar in both sexes: 21.4%, 95% CI: 10.5–32.3, 18/84 men included versus 18.0%, 95% CI: 10.2–25.8, 29/161 women included ($p = 0.5$). Most of the patients enrolled in the study were married or cohabiting (47.7%, $p = 0.2$), and the majority had a secondary education level (52.3%, $p = 0.06$), and were unemployed (56.5%, $p = 0.4$). Although only 11% of participants reporting their occupational status were in professions at high risk of exposure to *Cryptococcus* (rural tradesman and farmer), the rate of serum CrAg positivity in this group was relatively low, with only 5 positive cases out of 23 patients.

Remarkably, over three-quarters (76.9%) of patients with a positive serum CrAg test were on antiretroviral treatment (ART), in line with Congolese national HIV management guidelines. Patient characteristics are detailed in Table 1.

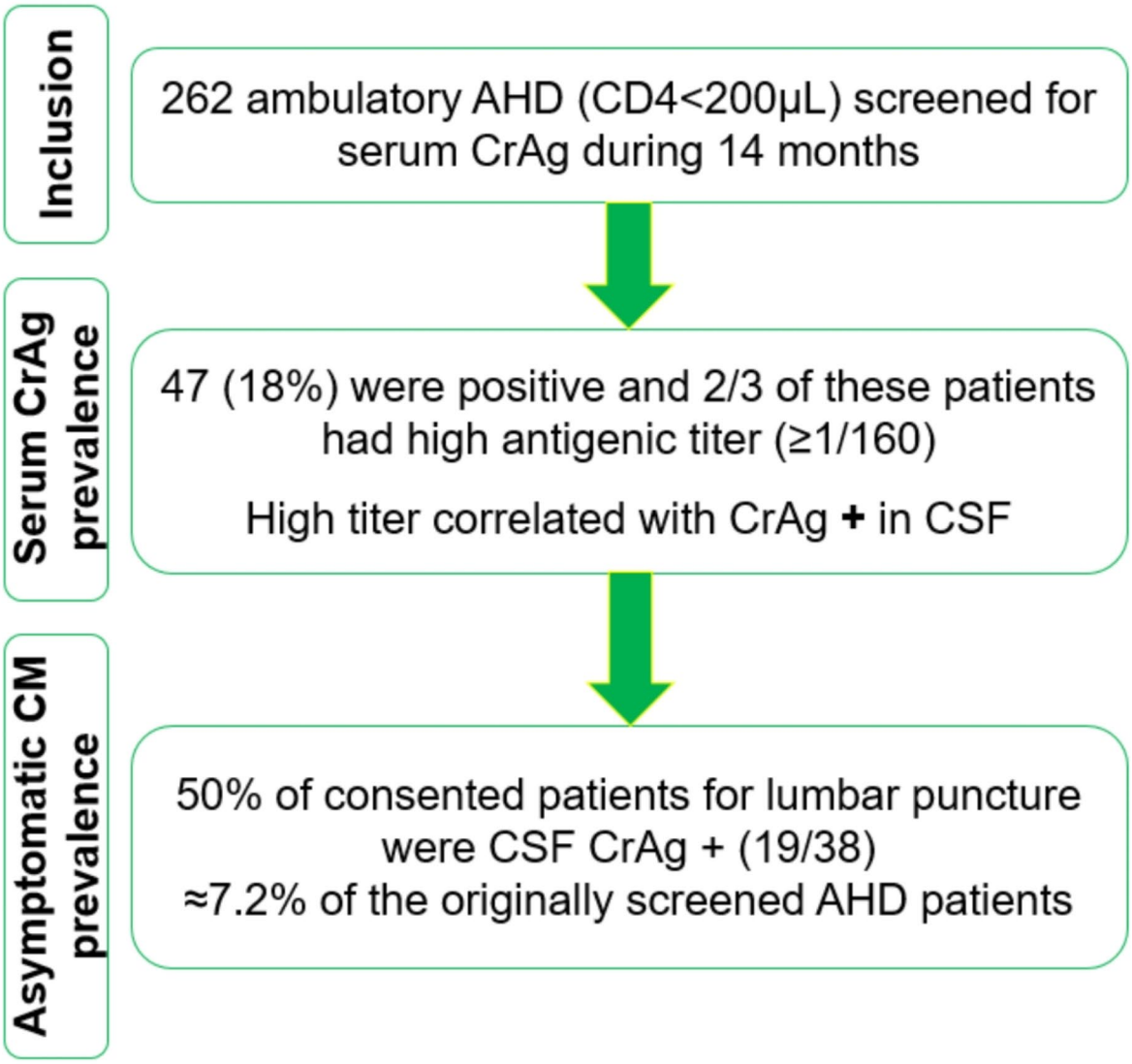

Fig. 1. Study flow: inclusion and screening of participants.

CD4 count versus HIV viral load in patients with a positive serum CrAg test - and in patients with asymptomatic cryptococcal meningitis

In this series of patients, median CD4 count of patients with a positive serum CrAg test was significantly lower than that of negative patients [64 (29–105) versus 95 (52–143) cells/µL, respectively, $p = 0.016$]. On the other hand, the median HIV viral load was approximately the same in the two patient groups [820 (40–55965) versus 849 (40–74977) copies/mL, $p = 0.74$, respectively] (Fig. 2).

On another note, meningeal invasion of *Cryptococcus* among patients with a positive serum CrAg test was independent of median CD4 count and HIV viral load (CD4 count: 51 versus 58 cells/µL, $p > 0.05$; viral load: 3116 versus 758 copies/mL, $p > 0.05$, positive and negative group patients respectively).

Considering the baseline CSF profile of patients with asymptomatic CM, it was predominantly clear (17 of 19) with a measured opening pressure < 20 cm H2O in all participants (100%). While direct Indian ink staining detected *Cryptococcus* in only 4 samples (21.1%), only 2 patients had a white blood cell (WBC) count greater than 10 (2/19), and around 1/3 of patients had trace amounts of protein in the CSF (31.6%). For the 6 patients for whom glycorrachia was measured, half had a value within the range and half had mild hypoglycorrachia.

MALDI-TOF MS identification, and genotypic profile of *C. neoformans* isolated from asymptomatic patients with cryptococcal meningitis

Of the 38 CSF samples collected from patients who consented to LP, only four (10.5%) were culture positive and subsequently characterized. Although having different identification scores and a sometimes dissimilar matching pattern, MALDI-TOF MS identified all four isolates as *Cryptococcus neoformans*. Similarly, the ITS2 sequence of

Characteristics[1]	Overall data (%)[2]	Serum CrAg		*p*-value	Crude OR (95% CI)
		Negative (%)	Positive (%)		
Demographic characteristics					
District of origin ($n=214$)				0.5	-
Lukunga	101 (47.2)	84 (48.3)	17 (42.5)		
Funa	47 (21.9)	39 (22.4)	8 (20.0)		
Mont Amba	36 (16.8)	26 (14.9)	10 (25.0)		
Tshangu	30 (14.1)	25 (14.4)	5 (12.5)		
Mean age ± SD (years) ($n=251$)	40.7 ± 13.2	41.5 ± 12.9	38.6 ± 13.9	0.1	-
Sex ($n=245$)				0.5	-
Female	161 (65.7)	132 (66.7)	29 (61.7)		
Male	84 (34.3)	66 (33.3)	18 (38.3)		
Marital status ($n=216$)				0.2	-
Single	81 (37.5)	62 (35.2)	19 (47.5)		
Married/cohabitating	103 (47.7)	85 (48.3)	18 (45.0)		
Divorced/widower	32 (14.8)	29 (16.5)	3 (7.5)		
Occupation ($n=209$)				0.4	-
Unemployed	118 (56.5)	94 (55.0)	24 (63.2)		
Low-risk employment[3]	68 (32.5)	59 (34.5)	9 (23.7)		
Risky employment[4]	23 (11.0)	18 (10.5)	5 (13.2)		
Education level attained ($n=214$)				0.06	-
None/primary	46 (21.5)	42 (24.0)	4 (10.3)		
Secondary	112 (52.3)	92 (52.6)	20 (51.3)		
Higher education/university	56 (26.2)	41 (23.4)	15 (38.5)		
Clinical history					
History of diabetes ($n=217$)	6 (2.8)	5 (2.8)	1 (2.5)	1	-
Alcohol ($n=217$)	60 (27.6)	51 (28.8)	9 (22.5)	0.4	-
Smoking ($n=217$)	26 (11.9)	19 (10.7)	7 (17.5)	0.2	-
Patients on ART ($n=216$)	172 (79.6)	142 (80.2)	30 (76.9)	0.6	-

Table 1. Demographic and clinical history of included patients. [1]According to the available cross-referenced data. [2]Column per cent calculated for each group. [3]Student, civil servants, taxi drivers, housewives and police/military officer. [4]Rural trader and farmer.

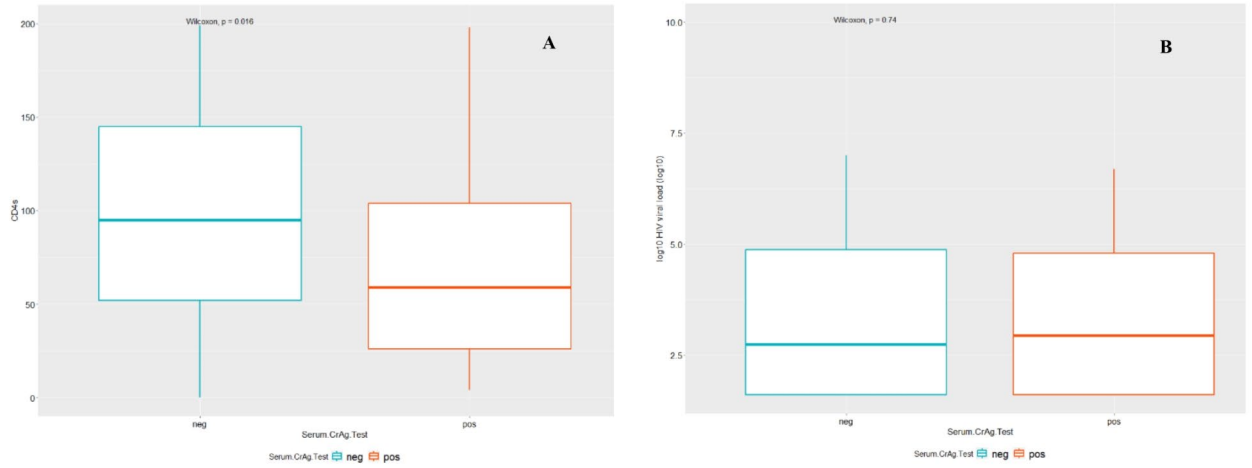

Fig. 2. (**A**) Boxplots of CD_4 counts in patients with advanced HIV disease by serum CrAg test; (**B**) HIV viral load by serum CrAg test.

all isolates matched with *Cryptococcus neoformans*; and the electrophoretic profile of serotype A (= *Cryptococcus neoformans*) was identified by performing multiplex serotyping PCR for all four samples. In further analysis, all isolates were identified as molecular type VNI applying the ISHAM MLST scheme, belonging to two different sequences type: ST93 (3/4, 75%) and ST63 (1/4, 25%).

Maximum likelihood phylogenetic reconstruction identified one main cluster among the investigated isolates, which grouped isolates from asymptomatic CM patients and those previously isolated from symptomatic patients (including a former DRC isolate from 1986), whereas a strain from an asymptomatic patient was weakly correlated with the main group (Fig. 3).

To understand the descent relationship between the genotype clusters analysed, a goeBURST analysis was performed. The minimum spanning tree generated showed two clonal complexes (CC) comprising the two groups of isolates involved in symptomatic (green and red hatched) and asymptomatic (yellow hatched) CM: CC1 (ST93, ST32 and ST31) and CC2 (ST53 and ST5). However, ST63 isolated from an asymptomatic patient with CM appeared as a singleton like the remnants of ST: ST69, ST4, and ST659 (Fig. 4).

Antifungal susceptibility testing of *C. neoformans* strains from CM asymptomatic patients

In vitro antifungal susceptibility testing was performed for the following three main antifungal agents: AMB, 5FC and FCZ, and all four study isolates showed MICs within the susceptibility ranges (Table 2).

Discussion

We carried out cross-sectional screening for serum and meningeal cryptococcosis in patients with asymptomatic advanced HIV disease in Kinshasa clinics. We then analyzed the genomic profile of the isolates involved in these patients in comparison with those previously described in symptomatic patients in the same region and clinics, in order to unearth the particularity of these strains.

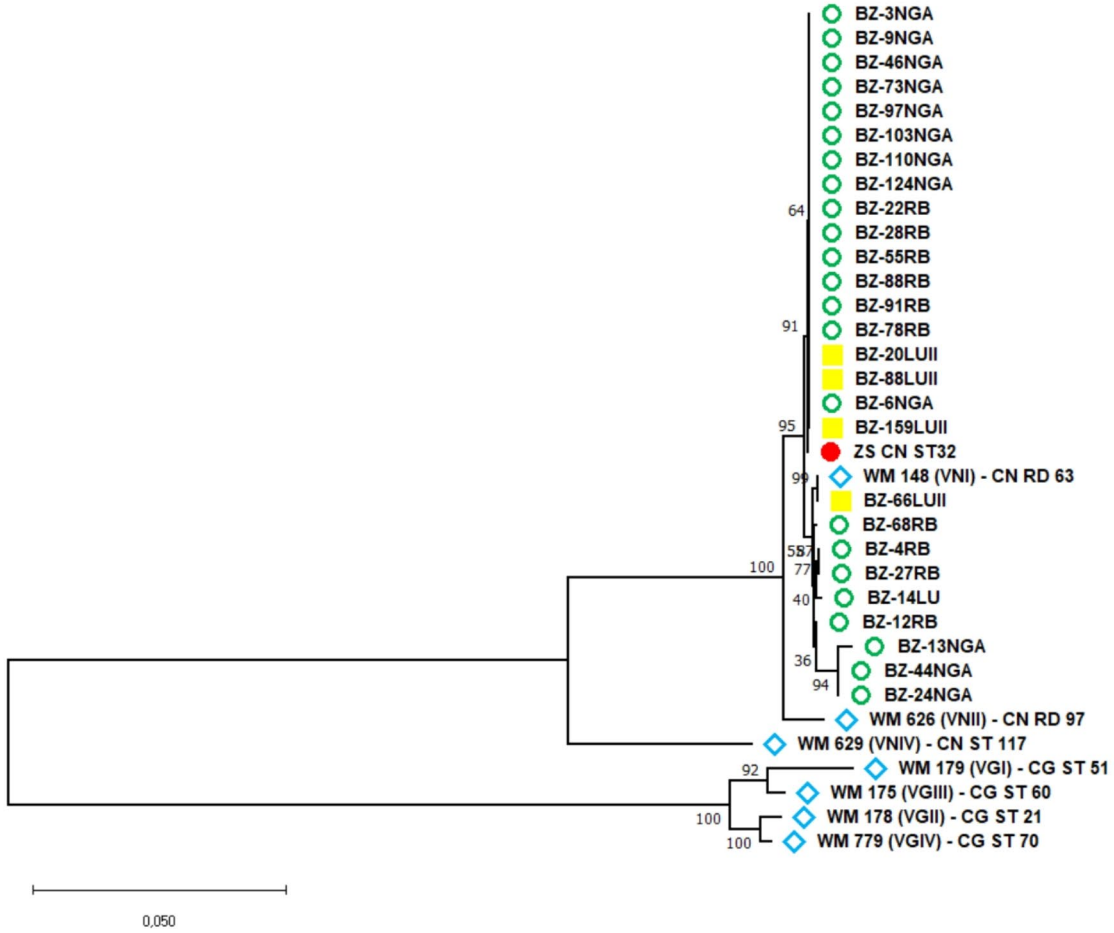

Fig. 3. Phylogenetic tree based on the concatenated sequences of the seven MLST loci: *CAP59*, *GPD1*, IGS1, *LAC1*, *PLB1*, *SOD1* and *URA5*. The numbers near the nodes represent the bootstrap values obtained for 1000 replicates. Isolates from PLHIV with asymptomatic (yellow chips) and symptomatic (green and red chips) CM are comparatively analysed. The blue chips symbolize WM reference isolates.

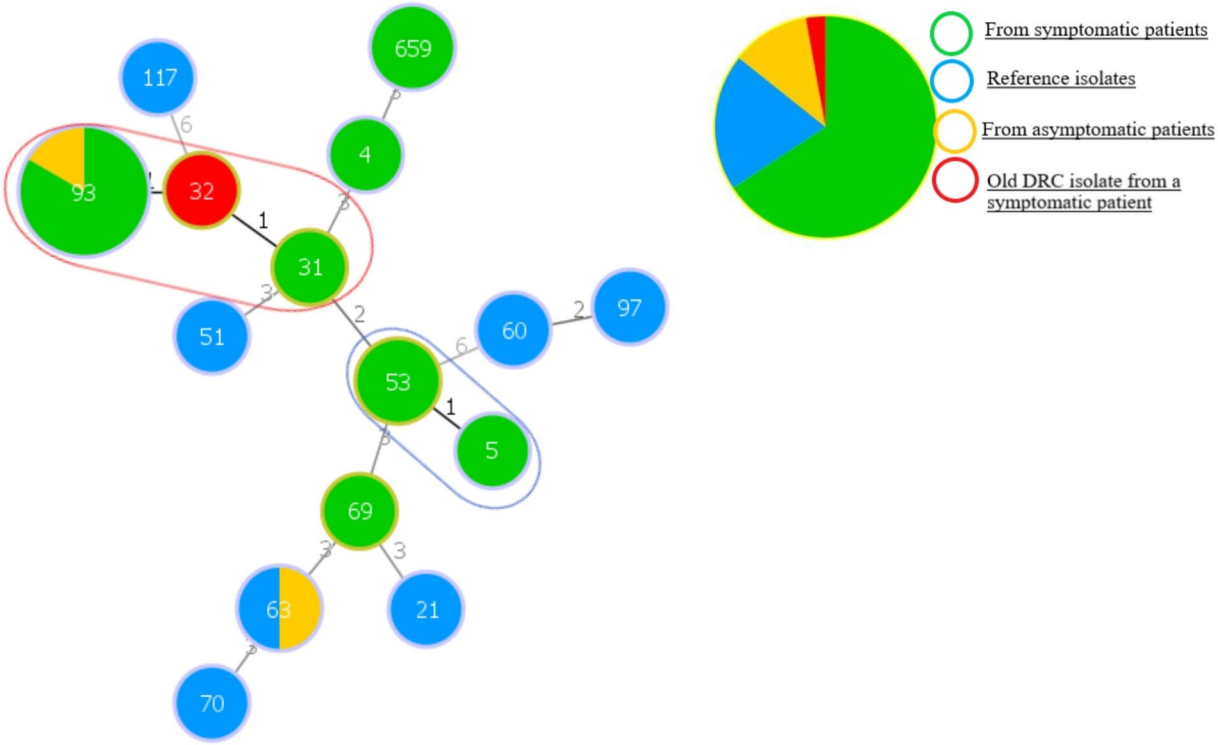

Fig. 4. Minimum spanning tree for *Cryptococcus neoformans* VNI isolates. Numbers inside the circles represent the ST code, whereas those on the branches are the number of different loci between the STs. The STs circled in red and blue represent the main clonal complexes, STs that are cluster linked by a single locus difference (=being identical at six other loci). Isolates from asymptomatic (yellow hatch) and symptomatic (green and red hatch) CM patients are comparatively analysed.

Isolate ID	AMB (CMI_{90} = mg/L) $S \leq 1$, $R > 1$	5FC (CMI_{50} = mg/L) $S \leq 8$, $R > 8$	FCZ (CMI_{50} = mg/L) $S \leq 8$, $R > 8$
20LU-BZ	0.5	8	1
66LU-BZ	1	1	8
88LU-BZ	1	2	8
159LU-BZ	1	2	8

Table 2. In vitro antifungal susceptibility testing results on strains isolated from the study.

Overall, 18% of included patients were serum CrAg positive and 50% of these had asymptomatic CM [i.e., CrAg positive cerebrospinal fluid (CSF)]. This represents 7.2% of the originally screened advanced HIV disease patients. Thirty years earlier in newly HIV-positive (antiretroviral-naïve) patients in Kinshasa (DRC), serum and then CSF CrAg were detected in 12.2% and 66%, respectively[10]. Compared to our study, the previous one focused on HIV-patients without taking into account the degree of HIV-induced immunosuppression (CD4 count). In addition, detection of cryptococcal antigens in that study was based on the latex agglutination test, which is less sensitive than the lateral flow assay used in our study[11]. Despite the improved in ART coverage among PLHIV (from 8 to 75%, 2010 to 2020), and the decrease in the number of new cases reported over the years in the DRC, the prevalence of cryptococcal antigenaemia has remained relatively high than the estimated mean global prevalence of 4.4% among advanced HIV disease patients[4,12]. Although the target population for each of the studies is not the same, the prevalence found in the present study remains worrying. About targeted patients, a Botswanan study clearly reported a higher prevalence in inpatients than in outpatients (21.9% versus 4.8%, respectively)[13].

In the present study, severe cryptococcosis was mainly identified in patients with high serum CrAg titers ($\geq$1:160), which is consistent with the literature[14,15]. Indeed, cryptococcal antigenemia is associated with a serious morbid condition in HIV-infected patients because of its natural tendency to progress to CM, both in antiretroviral-experienced and naïve PLHIV. The findings from Uganda[16,17]; Cameroon[18]; Botswana[19]; and Brazil[20], also support this.

In contrast to what has previously been described on the preferential CD4 threshold to be considered for screening given the resulting financial impact[19,21,22], our study showed no significant difference in the serum CrAg positive result between patients with less than 100 versus 100–200 CD4 cells/μL. This is in full agreement with the results found by Borges et al.., and James Wykowski et al., although in their studies, the absolute risk of all-cause mortality at 6 months was significantly higher in CrAg-positive patients with CD4 ≤ 100 cells/μL compared to 101–200 cells/μL. Increasing the CD4 count threshold for the reflex CrAg test to 200 cells/μL induces a high consumption of CrAg tests, also doubling the number of CrAg positive individuals identified[20,23]. This is therefore a deliberate choice of country prevention policy, as outlined in the WHO guidelines for management of cryptococcosis in HIV-positive patients[5,22].

Contrary to what has been described previously concerning the female protection against cryptococcal infections[24,25], in the present study, female with an average age of 40.7 ± 13.2 years old were relatively the most infected. Considering the included male number and the CrAg positive proportion compared to females, sex predominance was clearly neutral.

In this study, a low median CD4 count of 64 μL correlated with serum CrAg positivity in advanced HIV disease patients (versus 95 μL in serum CrAg negative patients), compared with a median HIV viral load. Although CD4 cell count and HIV viral load criteria are no longer applicable for eligibility for antiretroviral therapy, WHO guidelines and other studies identify CD4 cell count as a means of measuring a patient's immune status before starting ART or returning to care about treatment interruption, and as the best predictor of disease progression and risk of death, particularly in advanced HIV disease patients. It guides several of prophylactic and diagnostic interventions, including prioritisation of screening for opportunistic infections, such as cryptococcosis or tuberculosis. On the other hand, the main benefit of viral load measuring is to assess an individual's response to treatment following the abolition of the HIV-1 viral replication. Viral load suppression reduces morbidity and mortality in PLHIV and subsequent transmission of the virus. In the context of monitoring PLHIV, viral load testing is used as a preferred monitoring approach to detect treatment failure after ART initiation[26–28]. Our findings on this subject are in line with the literature.

One of the questions in this study was whether advanced HIV disease patients with asymptomatic CM are infected with *Cryptococcus* isolates of a different genetic profile than those infecting symptomatic patients in the same clinics in Kinshasa. Only a small number of strains were recovered from clinically inexpressive patients. Culture remains one of the key tests for diagnosing cryptococcosis from a sample containing viable *Cryptococcus*. However, various situations likely to deteriorate yeast viability (e.g. sub-optimal sample storage conditions) cause culture to lose its diagnostic value, while CrAg remain stable for a long time in blood and CSF, even after successful treatment. In the present study, the culture positivity rate was very low compared with that reported in the literature[29], even for CSF samples from patients with CrAg serum and CSF positive. The low storage temperatures applied in the study could be indexed. In addition, the screening context of the study is well suited to low fungal load situations, which would also be difficult to culture if an adequate fungal load is not achieved in inoculum. Sterile cultures have also been observed in patients on antifungals prior to sampling, which is clearly not the case in our asymptomatic patients.

Identification of *Cryptococcus* spp. strains by MALDI-TOF MS and the characterization of isolates using ITS2 sequencing and multiplex PCR serotyping showed that all isolates were *Cryptococcus neoformans* serotype A. This is consistent with the general profile of species identified among symptomatic PLHIV in Kinshasa clinics[9]. Furthermore, sequences type (STs) and phylogenetic analysis of isolates also showed strong similarity between isolates from symptomatic and asymptomatic CM patients, although one isolate (ST63) from an asymptomatic patient was weakly correlated with the main clusters. The similarity of the genetic profile in the two categories of patients could mean that they are the same nosological entity but at different stages of evolution, infra and clinical stages. Although the time that may elapse between the identification of *Cryptococcus* in an asymptomatic HIV-patient and the onset of general and meningeal signs/symptoms is well elucidated (about 22 days) by French et al.., the clinical and biological parameters governing this dynamic should be further documented[30].

To the best of our knowledge, this study is one of the first to compare STs involved in symptomatic versus asymptomatic CM in PLHIV. It is currently recognised that the phenotypic and physiological expression of *Cryptococcus* species differ according to the genotypic designation level of the yeast, from species to ST[3,31]. At the ST level, some lineages have phenotypes that make them more frequently or severely involved in infections than others. Thus, the Cryptococcal Optimal ART Timing (COAT) study classified the *C. neoformans* VNI STs analysed into 3 virulence groups, according to the survival time in humans and animals during infection: (1) high virulence (ST93, ST40, ST31); (2) intermediate virulence (ST5, ST77, ST93); and (3) low virulence (ST5, ST40, ST31)[31,32]. Although the association between virulence and ST clusters is widely described, individual STs have also been associated with high mortality and more severe clinical presentation[31]; for example, ST93 in Uganda[33]. The hypothesis that asymptomatic CM patients are infected with less virulent strains than those isolated from symptomatic patients is not clearly verified in this study; although one in four asymptomatic patients was infected with ST63, which is defined as an intermediate pathogenicity profile in the study by Vélez et al.[34]. Although it is a predominant ST in Europe and present in a few countries including DRC's neighbour Uganda, ST63 isolated in this study is the very first in DRC[34–36].

In contrast to the strains from symptomatic patients in Kinshasa clinics, which were sometimes resistant to the usual antifungal agents, the strains from asymptomatic patients were all susceptible to the antifungals tested. Although not unanimously agreed upon, the antifungal susceptibility profile of *Cryptococcus* isolates often does not correlate with their virulence, considering the therapeutic outcome of infected patients. In the cohort of Nascimento et al., no difference in the antifungal susceptibility of *C. neoformans* isolates from patients with favourable versus unfavourable outcomes was found[37]. Thus, the susceptibility of isolates from asymptomatic CM patients described here does not necessarily indicate a benign nature of these strains. Further studies on

the virulence factors of this isolates category, even in collaboration with other groups, and of course including a sufficient number of samples, could provide additional information.

In conclusion, serum and meningeal cryptococcosis remain highly prevalent among asymptomatic patients with advanced HIV disease in Kinshasa. The alarm bells are therefore ringing for the management of these vulnerable patients to be integrated into national HIV/AIDS and/or fungal disease control programs in the DRC, from active screening to pre-emptive treatment of positive patients. As in symptomatic PLHIV with cryptococcal meningitis, ST93 is mainly involved in asymptomatic patients in Kinshasa, although another ST of intermediate pathogenicity has been isolated in this group of patients.

Methods

Study design, patients and samples

A cross-sectional and analytical study was conducted in three Kinshasa public hospitals (DRC) supported by the non-profit organization for advanced HIV disease management, Doctors without Borders (Médecins Sans Frontières, MSF-Belgium). These were the following clinics: Centre Médical et Evangélique Révérend Luyindu, Centre Hospitalier Roi Baudouin 1er, and Centre Hospitalier Mère et Enfant de Ngaba. These clinics have the advantage of being located in three of Kinshasa's four districts, and each has an active cohort of 255, 570 and 622 PLHIV on ART, respectively. Between July 2020 and August 2021, patients seen in clinics for outpatient follow-up were consecutively screened for advanced HIV disease and serum cryptococcal antigens. Advanced HIV disease has been defined here primarily on the basis of a CD4 count below 200 cells/mm3. Patients with positive CrAg were approached for lumbar puncture (LP) to exclude *Cryptococcus* meningeal invasion, according to WHO guidelines. Overall, patient consent was sought twice: first for initial inclusion in the study (CD4 count and serum CrAg test), then for lumbar puncture and subsequent analyses. Cryptococcal antigenemia or cryptococcal meningitis (CM) was therefore established for positive patients and pre-emptive antifungal treatment was administered in line with WHO recommendations[5,6]. Ultimately, patients with positive CrAg test in both serum and CSF were retained for CM; those positive only in serum were considered to have bloodstream cryptococcosis. Positive CSF cultures were then characterized and tested for antifungal susceptibility testing with main antifungals. Any symptomatic patient or having a cryptococcosis history in the previous 12 months was not considered.

Biological analysis

The Alere PIMA CD4 analyzer was used for the CD4 counting of any patient before inclusion (Alere Inc., Waltham, MA, USA), and the HIV viral load (VL) was measured using the Abbott m2000 system as previously described[38,39]. Serum and CSF CrAg were detected using the CrAg LFA IMMY test, according to the manufacturer's instructions (Immuno-mycologic, Norman, OK, USA). All collected CSF samples were cultured on Sabouraud Dextrose Agar-Chloramphenicol medium at 30 °C for an average of 72 h, with incubation extended to two weeks for slow-growing samples (SDA-C, bioMérieux, Marcy-l'Etoile, France). Given the constraints relating to transporting samples from collection sites to the analysis laboratory, samples were inoculated one week after collection (on average), during which time they were kept cool (-10 to -20 °C). To further characterize the CSF from patients with asymptomatic CM, the following tests were performed using the standard techniques previously described[9]: glycorrachia, proteinorachia (Pandy test), WBC count and direct India ink staining.

MALDI-TOF MS identification, and molecular characterization of *Cryptococcus* spp.

Cryptococcus spp. identification was first performed using the Bruker MALDI-TOF MS system (Bruker Daltonics Bremen, Germany), followed by genetic characterization using serotyping multiplex PCR, ITS2 sequencing, and the International Society of Human and Animal Mycology (ISHAM) multilocus sequence typing (MLST) scheme, as previously described in the literature[40–43], and adapted in our previous papers[9,44].

Phylogenetic analysis

Phylogenetic analysis of concatenated sequences of the seven MLST loci was performed using MEGA v.6.06 software (http://www.megasoftware.net/features.html). 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. The sequences of isolates from the present study and those from previously described in symptomatic Congolese patients were analysed and compared together[9]. An old strain from a Congolese patient isolated in 1998 (formerly Zarian) and whose sequences were deposited in the MLST fungal database was also included in the analysis (https://mlst.mycologylab.org/). In addition, sequences of reference strains of *C. neoformans* and *C. gattii* species complexes were also inserted in the phylogenetic analysis as out-group for comparison (WM strains, whose sequences were also retrieved online from the MLST fungal database).

Furthermore, minimum spanning tree was generated using the geoBURST algorithm to determine the STs clonal complexes (CCs) representing linked and closely related clusters (https://online.phyloviz.net). We hence compared in the same spatial environment the ST profiles of isolates from the present study, reference strains, and those of isolates symptomatically infecting PLHIV in Kinshasa, according to earlier study[9].

Antifungal susceptibility testing

Minimum inhibitory concentrations (MICs) of major antifungal agents were determined according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) E.Def 7.3.1 procedure as previously described[45]. The interpretation criteria for amphotericin B (AMB) were those defined in the antifungals EUCAST breakpoint tables version 10.0: susceptible, ≤ 1 mg/L; resistant, > 1 mg/L. Not being defined in the EUCAST breakpoint tables, the fluconazole (FCZ) and 5-flucytosine (5FC) interpretation criteria were based

on the epidemiological cut-off values for in vitro antifungal susceptibility testing provided by the Clinical and Laboratory Standards Institute (CLSI) as follows: for both FCZ and 5FC, susceptible, ≤ 8 mg/L; resistant, > 8 mg/L[46].

Statistical analysis

Data were analyzed using R® version 4.2.1. Results of CrAg screening in the included patients were expressed as percentages [with their 95% confidence interval (CI)]. Categorical variables were compared using Pearson's chi-square test or Fisher's exact test as required. Continuous variables were summarised as mean ± standard deviation (SD) or median [and interquartile range (IQR)] as required and compared using Student's t-test or Wilcoxon rank-sum test to compare means or medians as required, respectively. Based on self-reporting, participants' occupations were classified into two groups according to exposure to cryptococcal ecological niches, in order to analyze exposure in relation to the presence of CrAg in patients' serum: (1) Low-risk employment (students, civil servants, cab drivers, housewives and police/military) and (2) High-risk employment (rural tradesmen and farmers). Viral load results whose target was not detectable (< 40 copies/µL) were rounded to 40 copies/µL for ease of quantitative analysis. Missing data were considered completely random, and the available data were analysed as such. A *p-value* of < 0.05 was considered to define statistical significance.

Ethical considerations

The Public Health School Ethics Committee of the Faculty of Medicine of the University of Kinshasa approved this study before implementation under the approval number *ESP/CE/071/2019*. All included patients were informed of the risks associated with the study and gave their written informed consent to participate, both during venipuncture and LP inclusion step, if applicable; in line with the Declaration of Helsinki. No personally identifiable information was collected. Data generated by the study were kept and handled by the research team alone.

Limitations of the methodological approach

In the study, samples were stored at very low temperatures depending on the equipment available in the sample collection clinics, sometimes for long periods before being sent to the copulation laboratory for analysis. This approach could be responsible for the unviability of most samples, justifying a low rate of positive cultures. The small number of strains obtained in this way is a major obstacle to the effective consideration of the related results.

Study limitations

The small number of strains isolated from asymptomatic patients included in this study means that no definitive conclusions can be drawn regarding the hypothesis put forward. A larger study including numerous strains from different regions in the same study design, also combining the virulence study of these strains in relation to that involved in symptomatic subjects will provide a better understanding of this question.

Data availability

All data generated or analyzed as part of this study have been fully integrated. ITS2 sequence data have been deposited in GeneBank and assigned the following accession numbers: strain 20LU-BZ= PP600226; strain 66LU-BZ=PP600227; strain 88LU-BZ= PP600228; strain 159LU-BZ= PP600229. Next Generation Sequencing raw data, MLST analysis results form and MALDI-TOF MS analysis results are available and accessible via the following link: https://drive.google.com/drive/folders/1jRWKDt8lS0YJctAuB7ovNxhRmp2H1iaa? usp=sharing.

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Author contributions

BZB, HSN and MM: designed the study. BZB: implemented the study and drafted the manuscript. BZB and RS: performed the biological analyses. BZB and DMK: performed statistical analyses and interpreted study results. AM, JAM and ME: collected biological data in the hospitals. GML and MPH: acquired funds and supervised the study. All authors reviewed and approved the latest version of the manuscript.

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Declarations

Competing interests
The authors declare no competing interests.

Additional information
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