Development and validation of quantitative PCR assays for HIV-associated cryptococcal meningitis in sub-Saharan Africa: a diagnostic accuracy study

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Summary

Background HIV-associated cryptococcal meningitis is the second leading cause of AIDS-related deaths, with a 10-week mortality rate of 25–30%. Fungal load assessed by colony-forming unit (CFU) counts is used as a prognostic marker and to monitor response to treatment in research studies. PCR-based assessment of fungal load could be quicker and less labour-intensive. We sought to design, optimise, and validate quantitative PCR (qPCR) assays for the detection, identification, and quantification of *Cryptococcus* infections in patients with cryptococcal meningitis in sub-Saharan Africa.

Methods We developed and validated species-specific qPCR assays based on DNA amplification of *QSP1* (*QSP1*A specific to *Cryptococcus neoformans*, *QSP1*B/C specific to *Cryptococcus deneoformans*, and *QSP1*D specific to *Cryptococcus gattii* species) and a pan-*Cryptococcus* assay based on a multicopy *28S rRNA* gene. This was a longitudinal study that validated the designed assays on cerebrospinal fluid (CSF) of 209 patients with cryptococcal meningitis at baseline (day 0) and during anti-fungal therapy (day 7 and day 14), from the AMBITION-cm trial in Botswana and Malawi (2018–21). Eligible patients were aged 18 years or older and presenting with a first case of cryptococcal meningitis.

Findings When compared with quantitative cryptococcal culture as the reference, the sensitivity of the *28S rRNA* was 98·2% (95% CI 95·1–99·5) and of the *QSP1* assay was 90·4% (85·2–94·0) in CSF at day 0. Quantification of the fungal load with *QSP1* and *28S rRNA* qPCR correlated with quantitative cryptococcal culture (R^2=0·73 and R^2=0·78, respectively). Both Botswana and Malawi had a predominant *C neoformans* prevalence of 67% (95% CI 55–75) and 68% (57–73), respectively, and lower *C gattii* rates of 21% (14–31) and 8% (4–14), respectively. We identified ten patients that, after 14 days of treatment, harboured viable but non-culturable yeasts based on *QSP1* RNA detection (without any positive CFU in CSF culture).

Interpretation *QSP1* and *28S rRNA* assays are useful in identifying *Cryptococcus* species. qPCR results correlate well with baseline quantitative cryptococcal culture and show a similar decline in fungal load during induction therapy. These assays could be a faster alternative to quantitative cryptococcal culture to determine fungal load clearance. The clinical implications of the possible detection of viable but non-culturable cells in CSF during induction therapy remain unclear.

Introduction

HIV-associated cryptococcal meningitis is the second leading cause of all AIDS-related mortality.[1] An estimated 152 000 cases of cryptococcal meningitis occur each year, leading to approximately 112 000 deaths.[2] Even with the recommended available antifungal regimens such as amphotericin B, flucytosine, and fluconazole, 10-week mortality is still 25–30%.[3,4]

Cryptococcus neoformans and *Cryptococcus gattii* species complexes are responsible for cryptococcosis in humans. *C neoformans* is the predominant causative organism, with

elevated morbidity and mortality in immunocompromised individuals, whereas *C gattii* is predominant in immunocompetent individuals.[5] *C neoformans* species complex can be characterised into serotype A (*C neoformans*), serotype D (*C deneoformans*), and A–D hybrids, and *C gattii* species complexes are subdivided into serotype B and serotype C, including *C gattii*, *Cryptococcus bacillisporus*, *Cryptococcus deuterogattii*, *Cryptococcus tetragattii*, and *Cryptococcus decagattii*.[6] At least three quantitative PCR (qPCR) assays have already been developed to discriminate these species on pure colony DNA;[7–9] however, these assays are limited in

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Research in context

Evidence before this study

We searched primary and review articles on PubMed, with the search terms cryptococcosis and cryptococcal diagnosis, up until Jan 15, 2023. Only results in English were included. Eight primary studies were identified that had either developed or validated quantitative PCR assays for *Cryptococcus neoformans* and *Cryptococcus gattii* in different specimens, such as fungal strains, mice tissue, and clinical samples (cerebrospinal fluid [CSF], sputum, and blood). However, most of these assays were not validated on clinical specimens (only fungal strains) and were qualitative (conventional PCR). One study was quantitative, but it was not validated on human samples, therefore potential use of the primer sets for monitoring patient prognosis is limited.

Added value of this study

We developed and validated qPCR and reverse transcriptase qPCR assays based on *QSP1* and *28S rRNA* that show good correlation with quantitative cryptococcal culture, which is the current gold standard for cryptococcal infection confirmation and is also a prognostic marker of the disease. Our assays enabled identification of the species involved (*C neoformans* vs *C gattii*) on initial CSF samples. We also showed good sensitivity of our designed assays when compared with quantitative cryptococcal culture. We were able to follow the decrease of the fungal load under optimal

treatment within the first 14 days of therapy. Our assays could be used in place of quantitative cryptococcal culture to improve turnaround times, which could help to decrease morbidity and mortality of cryptococcal meningitis.

Implications of all the available evidence

The WHO fungal priority pathogens list was released in 2022 to guide research, development, and public health action. One of the key areas identified for action was strengthening laboratory capacity and surveillance. *C neoformans*, which is the leading cause of cryptococcal meningitis in sub-Saharan Africa, is listed in the critical group of pathogens and therefore urgent attention to the pathogen is essential. Our qPCR assays could improve laboratory surveillance of cryptococcal meningitis due to *C neoformans* and *C gattii* species by reducing turnaround times and increasing laboratory efficiency. Identification and quantification on the initial CSF sample is the first step in designing new clinical studies of therapeutic strategies for cryptococcal meningitis. Our assays are currently used in our clinical laboratory to improve the diagnosis of cryptococcal meningitis in France. We recommend the use of our developed assays in mycology laboratories, to improve timely monitoring of patient response to treatment, and in future clinical trials at diagnosis, to identify patients with high fungal burden and evaluate different therapeutic strategies.

their diagnostic capacity because most of them are qualitative and not validated on clinical specimens following Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines.[10]

Methods used to diagnose cryptococcal meningitis include India ink staining, fungal culture, and cryptococcal polysaccharide capsular antigen (CrAg) detection. CrAg detection can be performed with lateral flow assays, which are easy and quick to perform.[11–14] However, CrAg assays are primarily qualitative and semi-quantitative and do not correlate with the fungal load under treatment, which could be important for the timely monitoring of patient prognosis. Kinetics of CrAg in patients on treatment cannot be used as a prognostic marker because they do not correlate well with a decline in quantitative cryptococcal culture. CrAg also remains positive even after patients have cleared colony-forming units (CFU) via quantitative cryptococcal culture, which limits the usefulness of CrAg testing in patient monitoring.[14,15] CFU counts using quantitative cryptococcal culture are performed in research studies to quantify the fungal load of culturable yeasts in the cerebrospinal fluid (CSF) and evaluate early fungicidal activity.[16,17] However, quantitative cryptococcal culture is fastidious and time-consuming. qPCR allows for DNA quantification and reverse transcriptase qPCR (RT-qPCR) allows for whole nucleic acid (WNA) amplification and evaluation of viability by detecting mRNA[18–21] and is a potential alternative method to monitor patient response to treatment.[20]

In this study, we aimed to develop and validate qPCR assays for the identification of *Cryptococcus* species using

CSF samples from patients presenting with cryptococcal meningitis.

Methods

Study design

We developed and evaluated four qPCR assays to identify *Cryptococcus* species or species complexes and quantify *Cryptococcus* load directly from CSF. We then validated our assays using CSF samples from participants enrolled in the AMBITION-cm trial in sub-Saharan Africa (ISRCTN72509687) to offer a quantitative molecular approach to monitor fungal load dynamics. This study was performed at Institut Pasteur (Paris, France). The AMBITION study compared a single high dose of liposomal amphotericin B (10 mg/kg of bodyweight) on day 1 plus 14 days of flucytosine (100 mg/kg per day) and fluconazole (1200 mg per day) to the reference treatment, which included amphotericin B deoxycholate (1 mg/kg per day) plus flucytosine (100 mg/kg per day) for 7 days, followed by fluconazole (1200 mg per day) for 7 days (control).

qPCR assays and primer design

Primers specific to *C neoformans* (serotype A), *C deneoformans* (serotype D), and *C gattii* species complex (serotype B or C) were designed using Primer3 version 4.0 and checked for secondary structures with the OligoAnalyzer Tool. Primer-BLAST was used to check for in-silico specificity. Two assays were developed, targeting the single copy gene Quorum sensing protein 1 (*QSP1*) and the multicopy gene

28S rRNA (figure 1). *QSP1* is a *Cryptococcus*-specific gene with no ortholog or paralogs outside of *C neoformans* and *C gattii* species complexes.[22] The *28S rRNA* gene is a repeated gene, a category of genes that is currently recommended to improve the sensitivity of the qPCR assay.[10] However, the number of copies can vary between isolates.[23] Therefore, for precise quantification, a unique gene (here *QSP1*) is preferred, when sensitivity of the detection is not an issue. The 28S rRNA assay was designed to capture the complete diversity within the *C neoformans* and *C gattii* species complexes in one single assay because designing a specific assay for each recently described species[6] is not cost-effective or relevant in routine care.

Three *QSP1* assays specific to serotype A (*QSP1*A), D (*QSP1*D), and B or C (*QSP1*B/C) were designed for identification and precise quantification (one copy corresponding to one cell; figure 1). The *28S rRNA* assay was designed to be pan-*Cryptococcus* and to improve sensitivity to detect low fungal loads as present in several copies in the genome. An RT-qPCR assay detecting *QSP1* mRNA using *QSP1*A primers was used to validate and check *Cryptococcus* viability in the clinical specimens. Indeed, mRNA is one of the most abundant transcripts in the *Cryptococcus* cells.[22] Because mRNA transcripts are more fragile than DNA copies, a decreased detection of mRNA as compared with DNA was considered as a proxy of dead yeasts, with only viable cells allowing the production of an increased quantity of mRNA (appendix p 3).

Primer specificity

The primers were tested on the *C neoformans* reference strain H99, with cells seeded in phosphate-buffered saline (simulated CSF) and in healthy blood samples at different concentrations and stored at –80°C for at least 48 h. This method was intended to mimic the storage conditions of the CSF specimens collected during the trial.[3] The assays were tested on all the *C neoformans* and *C gattii* species complexes, and a panel of 89 other species (appendix p 2) and 14 human DNA samples from patients undergoing toxoplasma screening in blood without cryptococcosis to rule out cross-reactivity. The primer sequences are shown in the table.

Nucleic acid extractions

For optimisation experiments (appendix p 3), WNA (representing DNA + RNA) was extracted from phosphate-buffered saline (PBS) seeded with H99 cells (simulated CSF) using the MagNA Pure 96 Instrument (Roche Diagnostics, Mannheim, Germany) and Viral NA Large Volume Kit (Roche Diagnostics). We used the DNA Blood LV 1000 (Roche Diagnostics) and the Pathogen Universal 1000 (Roche Diagnostics) kits for DNA extraction and compared their cycle quantification (Cq) values. Different pretreatment conditions were tested, including untreated (control), bead beating, adding 50 μL of proteinase K and 10 min incubation at 65°C, and a combination of bead beating and adding proteinase K.

For patient CSF screening, WNA was extracted from frozen CSF pellet obtained by centrifugation of 1 mL of CSF.
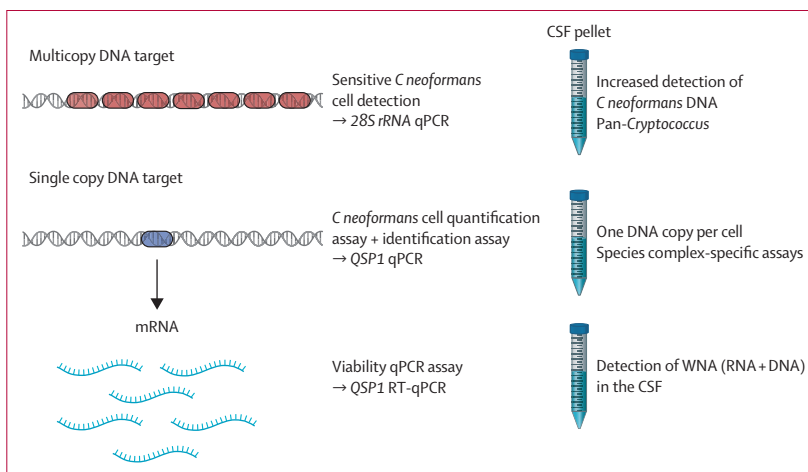
Figure 1: Schematic representation of the assays used in this study
C neoformans=Cryptococcus neoformans. CSF=cerebrospinal fluid. qPCR=quantitative PCR. RT-qPCR=reverse transcriptase quantitative PCR. WNA=whole nucleic acid.

The Pathogen Universal 1000 was used for extraction with 100 μL of elution. The RNA Process Control Kit (Roche Diagnostics) was used as an internal control in RT-qPCR, and a DNA internal control kit (DICR-CY5, Diagenode, Seraing, Belgium) was used in qPCR runs, with a defined virus quantity directly added in the sample before extraction, as recommended.[10]

See **Online** for appendix

qPCR and RT-qPCR

A Light Cycler 480 Instrument II (Roche Diagnostics) was used for all qPCR and RT-qPCR amplification and Cq analyses. A Light Cycler 480 Probes Master kit (Roche Diagnostics) was used for all qPCR reactions, and a TaqMan Fast Virus 1-Step Master Mix kit (Thermo Fisher Scientific, Waltham, MA, USA) was used for all RT-qPCR reactions. Final concentrations of 0·5 μM (primer) and 0·2 μM (probe) were used for *QSP1*, and of 0·4 μM (primer) and 0·2 μM (probe) for *28S rRNA* (table). qPCR reactions were run as follows: 95°C for 10 min, and 50 cycles of 95°C for 10 s, 58°C for 30 s, and 40°C for 30 s, with an additional initial step at 50°C for 5 min for RT-qPCR. All Cq values over 40 were considered negative. All qPCR assays were performed blind to quantitative cryptococcal culture results and all clinical information.

To quantify *QSP1*A expression, 10^6 H99 cells were heat-killed (65°C for 2 h) and exposed to 1 mM of H_2O_2 for 1 h with stationary phase yeasts as control and extracted as above. QSP1 mRNA expression was compared to that of actin (*ACT1*) considering the efficiency (*E*) of both assays (*QSP1, E*=2; *ACT1, E*=1·92).[24]

Ten-fold dilutions from the strain H99 cells (*QSP1*A), WM276 cells (*QSP1*B/C), and JEC21 cells (*QSP1*D) were used to create a standard curve, and extrapolation was used to determine the absolute number of *Cryptococcus* cells present in the sample (appendix p 3).

A semi-quantitative lateral flow CrAg test (CryptoPS; Biosynex, Strasbourg, France) was performed on plasma according to the manufacturer's instructions.[25,26]

	Sequence (5'-3')	Melting temperature (°C)	Length (bp)	Amplicon length (bp)
H99_QSP1_RNA_F2	ACCACTCTTTTCACTGCTG	56·2	19	105
H99_QSP1_RNA_R2	GGCGCCGAAGTTGTTAG	56·6	17	105
H99_QSP1_RNA_P2	CTTGTCCTCATCGCCCCGGCCCTC	71·9	24	105
WM276_QSP1_F2	ACCACACTTTTCACTGCCG	56·2	19	105
WM276_QSP1_R2	GGCACCGAAGTTCTGAG	56·6	17	105
WM276_QSP1_P3	CTTGTCCTCATCGCCCCTGCCCTC	65·5	24	105
JEC21_QSP1_F2	ACCACCCTTTTCACTGCTG	56·2	19	105
JEC21_QSP1_R2	GGCGCCGAAGTTCTGAG	56·6	17	105
JEC21_QSP1_P2	CTTGTCCTCATCGCCCCGGCCCTC	71·9	24	105
AMB_28S rRNA_F3	GCAGGTCTCCAAGGTGAA	52·8	18	137
AMB_28S rRNA_R4	CCAGCTTCCTTCCGTCAA	55·0	18	137
AMB_28S rRNA_P4	TTGGCTCTAAGGGTTGGGTGCGTCGGG	67·7	27	137

H99 is a reference strain of *Cryptococcus neoformans*; WM276 is a reference strain of *Cryptococcus gattii*; and JEC21 is a reference strain of *Cryptococcus deneoformans*. F represents a forward primer; R represents a reverse primer; and P represents a probe.

Table: Species-specific primer and probe sets that were designed and used in this study

Study participants and samples

We collected samples from 209 participants with cryptococcal meningitis from Botswana (n=85) and Malawi (n=124) enrolled in the AMBITION-cm randomised controlled trial. Patients aged 18 years or older presenting with a first episode of cryptococcal meningitis, which was diagnosed with India ink or CrAg, were enrolled in the study. For this qPCR analysis, samples collected from patients presenting at Princess Marina Hospital (Gaborone, Botswana) and Blantyre Central Hospital (Blantyre, Malawi) in 2018–21 were used.[3] CSF samples collected at day 0 (baseline), day 7, and day 14 post antifungal treatment initiation were tested with quantitative cryptococcal culture[27] and qPCR and RT-qPCR assays. Ethical approval was obtained from the University of Botswana Institutional Review Board and the University of Malawi Research and Ethics Committee and national regulatory bodies. Written informed consent was sought from participants for the initial AMBITION study; for those who did not have the capacity to consent, consent was sought from their next of kin.[3,27]

Statistical analysis

GraphPad Prism 9.4.0 was used for statistical analysis and data visualisation. Diagnostic accuracy outside sensitivity has not been calculated due to the lack of testing of patients without cryptococcal meningitis. Diagnostic measures were compared between the assays using Bland-Altman tests. Missing data were not included in the statistical analyses. Sample size was determined for the clinical study but was not calculated for our analyses because there were no anticipated results available in the literature and previous studies. Kruskal-Wallis tests were used to compare means between three or more matched groups when the data were not normally distributed, and Mann-Whitney tests were used for comparison of two groups when the data were not normally distributed. For the analytical specificity of the assays, the fold change ratio of the strains relative to the

reference strain was determined. A ratio of 0–0·3 indicated no amplification to poor amplification, 0·4–0·6 indicated low amplification, and 0·7–1·0 indicated good to very good amplification. $p < 0.05$ was considered to indicate a statistically significant difference.

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

The *28S rRNA* assay amplified all *C neoformans* and *C gattii* species complexes with ratios of 0·46–1·00 depending on the species tested. Species complexes other than *C neoformans* or *C gattii* were not amplified or were very poorly amplified with ratios of 0·01–0·33 (appendix p 2). No cross-reaction with non-*Cryptococcus* species complex strains (100% analytical specificity) was observed.

All *QSP1* assays (*QSP1*A, *QSP1*B/C, and *QSP1*D) were tested on the same 93 fungal species and did not amplify any of the species (appendix p 1). The *QSP1*A assay amplified serotype A DNAs with a ratio of 0·61–1·00, with the exceptions of VNII_T4, VNII_8, and VNBII_Bt1, for which amplification was lower, with a ratio of 0·12–0·27. The *QSP1*A assay was able to perfectly amplify VNIII (AD hybrid) with a ratio of 1, but not *C deneoformans* and *C gattii* species (appendix p 2). The *QSP1*D assay amplified *C deneoformans* and VNIII (AD hybrid) with a ratio of 0·67–1·00, but not *C neoformans* and *C gattii* species. The *QSP1*B/C assay allowed amplification of *C gattii* species, mainly *C gattii* sensu stricto, *C deuterogattii*, and *C tetragattii* (VGI, VGIV, and VGII), with ratios greater than 0·72. However, *C bacillisporus* DNAs were amplified with ratios of less than 0·1. The *QSP1*B/C assay did not amplify *C neoformans* and *C deneoformans* DNA.

To optimise and validate our qPCR assays, we first used spiked samples. The limit of detection for the *QSP1*A assay was 50 genomes per reaction and for the *28S rRNA* assay was one genome per reaction. The efficiency of the *QSP1*A assay was 1·98 (slope –3·347, r^2=0·998), and of the *28S rRNA* assay was 2·08 (slope –3·141, r^2=0·993; appendix p 3). For comparison of pre-extraction procedures, amplification of untreated (control) condition after freezing gave a significantly lower Cq value than the other conditions, including bead beating ($p < 0.0001$), addition of proteinase K (p=0·030), and a combination of bead beating and addition of proteinase K ($p < 0.0001$; appendix p 3) suggesting better extraction efficiency. There were no statistically significant differences between the two extraction protocols (Pathogen Universal and DNA Blood) of CSF tested on the MagNA Pure 96 Instrument (appendix p 3).

Upon screening of clinical CSF samples from baseline in 209 participants (demographic characteristics are provided in the appendix p 4), including 85 from Botswana and 124 from Malawi, we found a 67% prevalence (95% CI 55–75) of *C neoformans* in Botswana and 68% prevalence
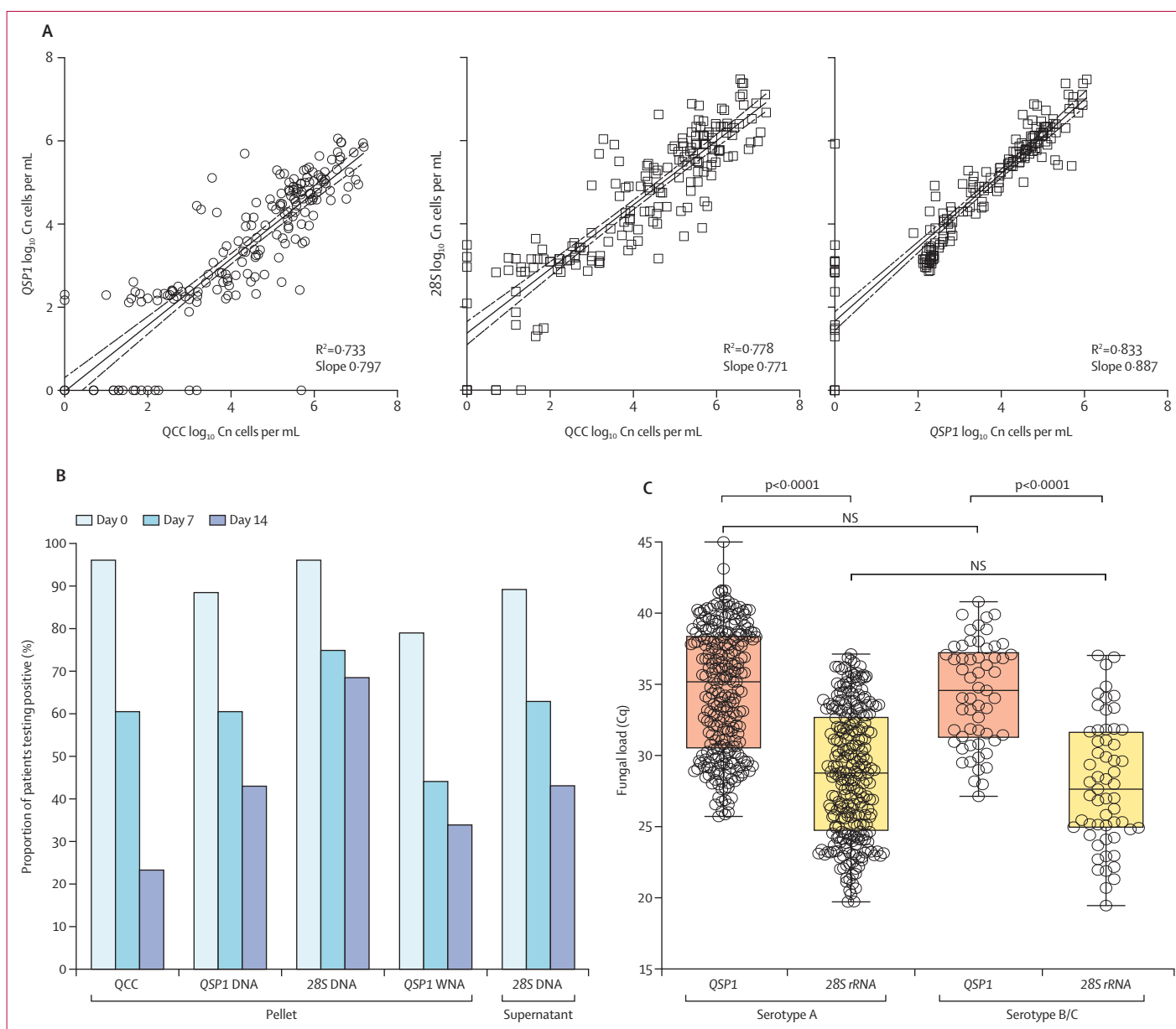
Figure 2: **Comparison of *QSP1* and *28S rRNA* assays to QCC and description of the proportion of positive quantitative tests in the cohort**
(A) Correlation between *QSP1* quantification (log$_{10}$ Cn cells per mL) and QCC (log$_{10}$ Cn cells per mL), between *28S rRNA* quantification (log$_{10}$ Cn cells per mL) and QCC, and between *28S rRNA* and *QSP1* using CSF pellet results at baseline (before starting antifungal treatment). (B) Proportion of CSF pellet and supernatant sample fractions amplified by *QSP1*A, *QSP1*B/C (combined in the figure as *QSP1*), and *28S rRNA* assays at day 0 (baseline), day 7, and day 14 of antifungal treatment. (C) Fungal loads in CSF at day 0 with *QSP1* and *28S rRNA* assays in *C neoformans* and *C gattii*. The boxes show median and IQR, and the whiskers show the range. Cn=*Cryptococcus neoformans* or *Cryptococcus gattii*. Cq=quantification cycles. CSF=cerebrospinal fluid. NS=not significant. QCC=quantitative cryptococcal culture. WNA=whole nucleic acid.

(57–73) in Malawi. The prevalence of *C gattii* species complex was higher in Botswana (21%; 14–31) than in Malawi (8%; 4–14). Ten (12%) of 85 samples in Botswana and 29 (23%) of 124 samples in Malawi could not be serotyped due to low fungal load (*QSP1* negative, *28S rRNA* positive). No *C deneoformans* cryptococcal meningitis was detected in both countries. Of note, three (4%) samples were missing at baseline in Botswana and one (1%) was missing at baseline in Malawi.

We compared the quantification obtained in CSF pellet samples from *C neoformans* and *C gattii* cryptococcal meningitis using *QSP1* and *28S rRNA* qPCR assays based on Cq values. There was no statistically significant difference in the initial fungal loads (expressed as Cq) of *C neoformans* and *C gattii* cryptococcal meningitis with the *QSP1* assay (35·18 [IQR 30·45–38·36] *vs* 34·57 [31·19–37·32], respectively) or the *28S rRNA* assay (28·77 [24·66–32·77] *vs* 27·64 [24·87–31·73], respectively; figure 2C). Similarly, no
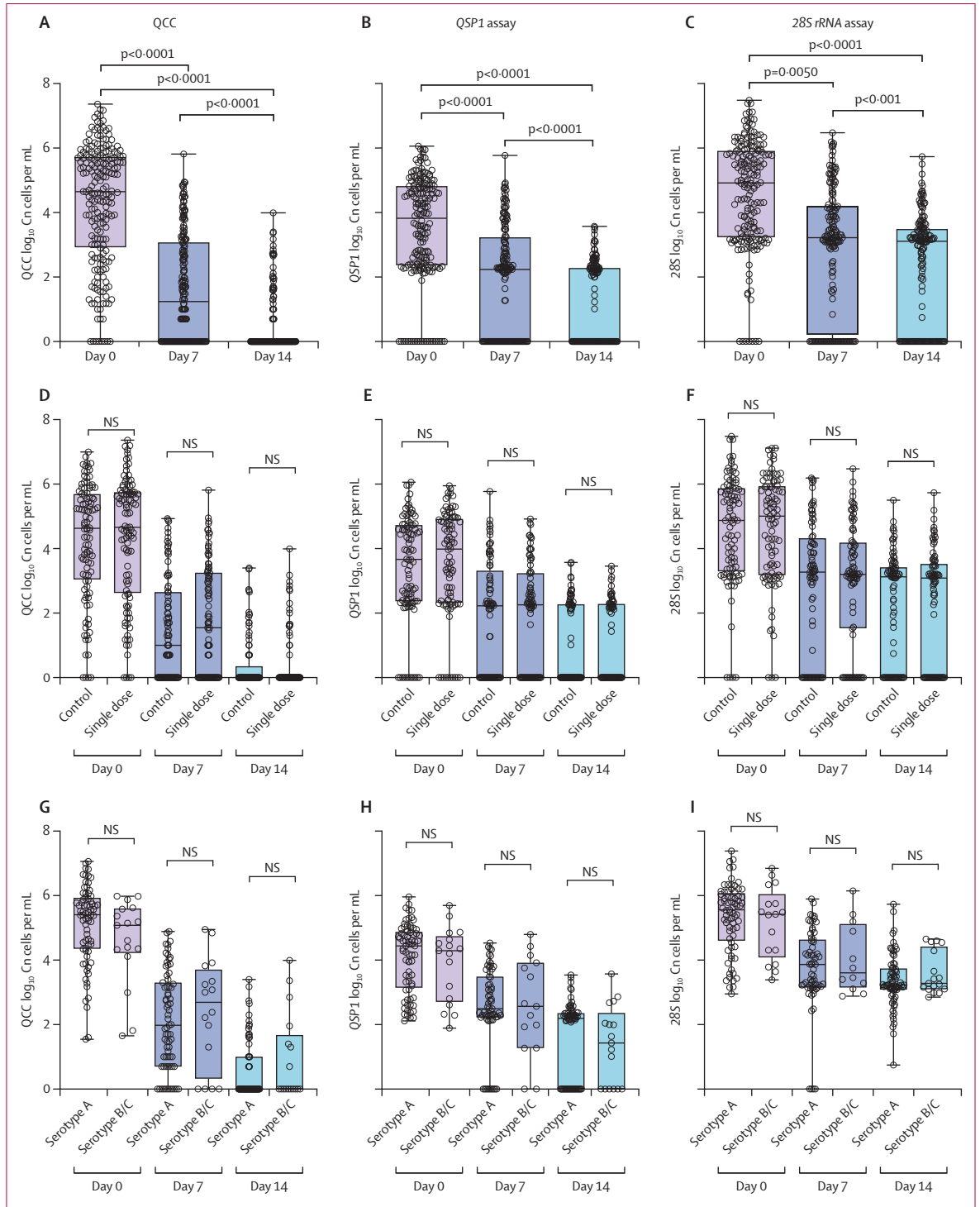
Figure 3: **Cryptococcal fungal load quantification**
Cryptococcal fungal load was quantified at day 0 (baseline), day 7, and day 14 in the cerebrospinal fluid of participants by QCC, *QSP1* assay, and *28S rRNA* assay in total populations (A–C) and in the control or single-dose regimens (D–F). Serotype-specific fungal load was quantified at day 0 by QCC, *QSP1* assay, and *28S rRNA* assay (G–I). The boxes show median and IQR, and the whiskers show the range. Cn=*Cryptococcus neoformans* or *Cryptococcus gattii*. NS=not significant. QCC=quantitative cryptococcal culture.
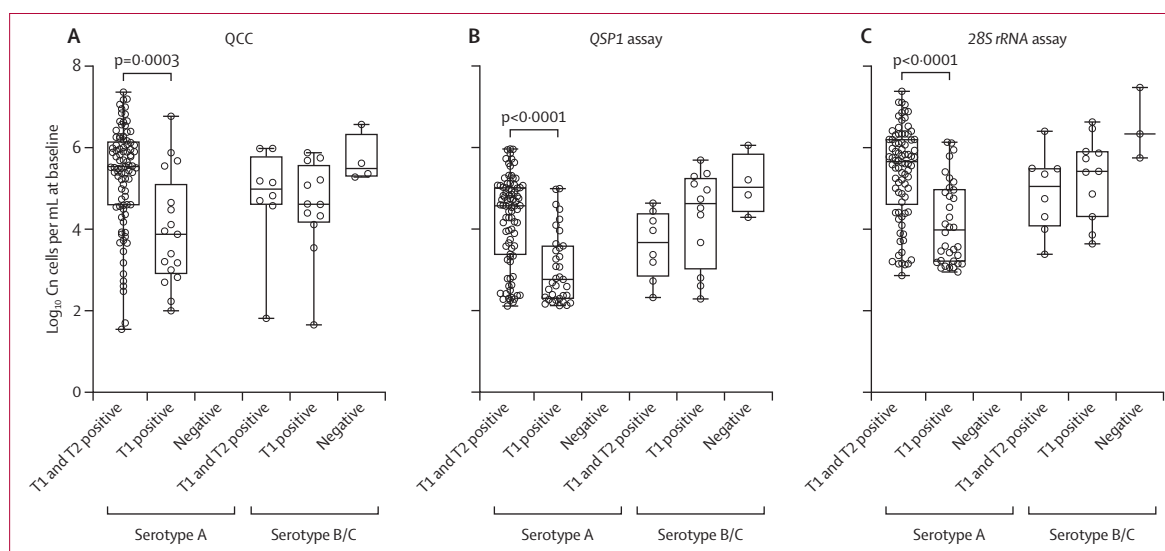
Figure 4: Quantification of the fungal load in the cerebrospinal fluid of participants with a positive T1 and T2 band, a positive T1 band, or who were negative in CryptoPS testing in plasma at baseline (day 0)

Quantification is shown for serotype A and B/C with QCC (A), *QSP1* assay (B), and *28S rRNA* assay (C). The boxes show median and IQR, and the whiskers show the range. Cn=*Cryptococcus neoformans* or *Cryptococcus gattii*. QCC=quantitative cryptococcal culture.

significant differences were observed for quantification by quantitative cryptococcal culture, *QSP1* assay, and *28S rRNA* assay with absolute yeast number (figure 3G–I). The differences in Cq value (ΔCq) between *QSP1* and *28S rRNA* assays for each CSF were significant: ΔCq of 5·71 (IQR 5·09–6·32), reflecting about 50 copies of *28S rRNA* per genome in *C neoformans*, and ΔCq of 6·55 (5·75–7·78), reflecting about 100 copies of *28S rRNA* per genome in *C gattii* (figure 2C). A similar finding was observed in the DNA extracted from strains during previous optimisations (appendix p 3).

We quantified DNA using *QSP1* assays and *28S rRNA* assays in CSF pellets and in CSF supernatant. We also quantified WNA (RNA plus DNA) in the CSF pellet as a potential proxy of the viability of the yeasts present in the pellet (n=110). At baseline in CSF pellets, the proportion of positivity with DNA was higher with quantitative cryptococcal culture (200 [95%] of 208 participants positive) and *28S rRNA* assay (177 [94%] of 184), as compared with the *QSP1* assay, with which 163 (88%) of 184 were positive (figure 2B). At day 14, the *QSP1* and *28S rRNA* assays showed an increased proportion of positive samples (43% and 69%, respectively) compared with quantitative cryptococcal culture (23%).

At baseline in CSF pellets, the sensitivity of the *28S rRNA* assay was 98·2% (95% CI 95·1–99·5) and of the *QSP1* assay was 90·4% (85·2–94·0) when compared with quantitative cryptococcal culture as the reference standard. Indeed, three CSF samples that were negative on quantitative cryptococcal culture were found to be positive with the *28S rRNA* and *QSP1* assays. The sensitivity was 98·0% (94·4–99·5) for the *28S rRNA* assay and 93·0% (87·9–96·0) for the *QSP1* assay when compared with the India ink results. Of note, 27 and

16 CSF samples that were negative on India ink were found to be positive with the *28S rRNA* and *QSP1* assays, respectively.

Detection of *28S rRNA* DNA in CSF supernatant samples gave a lower proportion of positivity compared with CSF pellet samples, with a maximum difference at day 14 (43% in supernatant *vs* 69% in pellet; figure 2B). *QSP1* WNA positivity was lower compared with *QSP1* DNA in CSF pellet samples at all timepoints but was still more positive at day 14 than in samples that were quantified with quantitative cryptococcal culture (34% for *QSP1* WNA *vs* 23% for quantitative cryptococcal culture). A Bland-Altman analysis showed an agreement between quantitative cryptococcal culture and *QSP1* or *28S rRNA* with a ratio bias of 1·24 (SD 0·28) and 0·90 (SD 0·24), respectively (appendix p 5).

We compared the quantification of fungal load with quantitative cryptococcal culture and *QSP1* and *28S rRNA* qPCR assays in CSF pellet samples at baseline. We found strong positive correlations between the three assays (figure 2A).

QSP1 and *28S rRNA* assays showed a significant decrease in cryptococcal fungal load between baseline and day 7 (both p<0·0001), between day 7 and day 14 (p<0·0001 for *QSP1* and p=0·0050 for *28S rRNA*), and between baseline and day 14 (both p<0·0001; figure 3B, C), which was similar to the decrease observed with quantitative cryptococcal culture (figure 3A). Of note, fungal load dynamics were similar in patients from each treatment group, thereby suggesting a similar fungicidal activity of the two strategies (figure 3D–F).

Of the 151 participant plasma samples that were determined as serotype A or B/C, and which were tested with CryptoPS at baseline, 98 (65%) had a positive T1 and T2
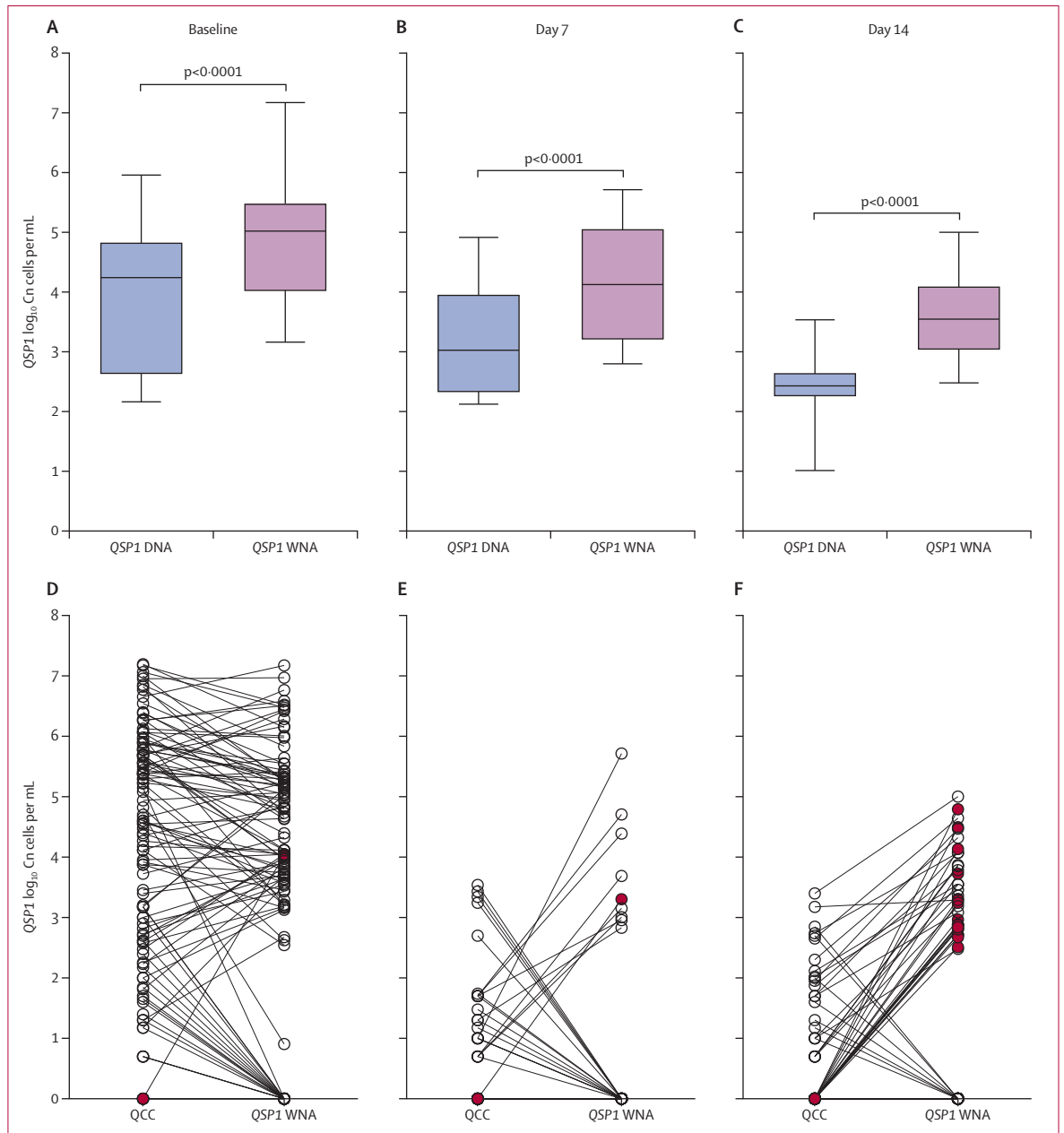
***Figure 5:* QSP1 DNA and WNA quantification in CSF pellet and comparison between QCC and *QSP1* WNA load**
QSP1 DNA (qPCR) and WNA (RT-qPCR) were quantified in CSF pellet at baseline (day 0; A), day 7 (B), and day 14 (C) using *QSP1* assays. The boxes show median and IQR, and the whiskers show the range. *QSP1* WNA load was compared with QCC at baseline (D), day 7 (E), and day 14 (F). Red circles are participants with negative QCC and positive *QSP1* WNA detection. Cn=*Cryptococcus neoformans* or *Cryptococcus gattii*. CSF=cerebrospinal fluid. QCC=quantitative cryptococcal culture. qPCR=quantitative PCR. RT-qPCR=reverse transcriptase quantitative PCR. WNA=whole nucleic acid.

band, 49 (32%) had a positive T1 band, and four (3%) were negative. Negative CryptoPS tests were identified in serotype B/C infections (4/24, 17%), whereas all serotype A participants had a positive CryptoPS test in the plasma at baseline. In serotype B/C, four samples were negative with CrAg but positive with quantitative cryptococcal culture (figure 4A), three were negative with CrAg but positive with the *QSP1* assay (figure 4B), and three were

negative with CrAg but positive with the *28S rRNA* assay (figure 4C).

We also wanted to determine whether the DNA detected and quantified in CSF came from viable yeasts using our *QSP1* assay with a reverse transcriptase step (RT-qPCR) allowing the detection and quantification of *QSP1* mRNA and DNA. We first validated that dead cells in vitro had a decreased expression of *QSP1*A as compared with *ACT1*

and a stationary phase control (appendix p 3). Using the H99 WNA, we found an increased quantification with RT-qPCR (median Cq 26·55 [IQR 22·86–32·59]) compared with qPCR (30·61 [27·46–36·61], p<0·0001; appendix p 3).

In patients, we found an increased QSP1A WNA quantification using RT-qPCR as compared with qPCR at all timepoints (baseline, day 7, and day 14, p<0·0001; figure 5A–C). An increase of between 0·8 (baseline) and 1·1 (days 7 and 14) $\log_{10}$ C neoformans cells was observed with WNA, showing that QSP1A mRNA was present in a higher quantity (around 10-times higher) than DNA. This finding suggested that yeast cells detected in the CSF pellet were viable, resulting in the detection of more QSP1 mRNA than DNA, and showing active expression of the QSP1 gene.

We then compared quantitative cryptococcal culture load and QSP1 WNA load in individual samples. Quantitative cryptococcal culture was negative and QSP1 WNA positive in one sample at baseline and day 7, and in 17 samples at day 14 (figure 5D–F, red circles). We thus identified patients with a negative CSF culture but a positive QSP1 RT-qPCR assay, suggesting that viable but non culturable yeasts were observed in the CSF during meningitis. The week 10 mortality of these patients was 25·1% (51/203).

Discussion

In this study, several qPCR and RT-qPCR assays (QSP1A, QSP1B/C, and QSP1D, and 28S rRNA assays), allowing Cryptococcus load quantification and identification of C neoformans, C deneoformans, and C gattii species complexes, were designed, optimised, and clinically evaluated using samples from the AMBITION-cm trial.[3] The analytical specificity and the clinical sensitivity have been validated and show optimal performance, following dedicated MIQE guidelines.[10] We report optimal qPCR efficiencies of around 100% for all assays. We recommend using the 28S rRNA assay as a screening assay and, when positive, identifying the species and quantifying the fungal load with the three QSP1 qPCR assays.

Of note, we identified a high burden of C gattii species complex infections in Botswana (21%), as already reported (30% for Botswana).[28,29] No C deneoformans (serotype D) cases were detected, as already shown.[30]

The designed QSP1 and 28S rRNA assays showed excellent correlation with quantitative cryptococcal culture quantification. Quantitative cryptococcal culture is the current gold standard for fungal load quantification, with a reported 94·2% sensitivity in South Africa and Uganda.[13] We observed a gap between quantitative cryptococcal culture and DNA detection, with an increased number of participants detected with the QSP1 and 28S rRNA qPCR assays, suggesting that yeasts that were unable to grow on culture media or that were dead could be detected by qPCR. Nevertheless, participants had lower fungal loads at day 7 or day 14 (or both) than at baseline, demonstrating the early fungicidal activity of the two different treatment groups of the AMBITION study.[3] This finding is important because a decrease of the fungal load and fungicidal activity could

be determined using qPCR quantification instead of quantitative cryptococcal culture.[17]

By using WNA amplification with our QSP1 assay, we validated that most of the yeasts detected, even at day 14, were viable, with an increased QSP1A WNA detection compared with DNA. We thus detected significantly more QSP1 WNA than DNA in participants with negative quantitative cryptococcal culture results at baseline, day 7, and day 14, suggesting that living yeasts were present in the CSF despite a negative culture. These cells are suggestive of viable but non-culturable cells (VBNCs), corresponding to viable living yeasts that are not capable of growth on agar medium. This phenotype is known to exist in Cryptococcus yeasts cells and has been identified and characterised by our team.[31,32] This is a major finding because RT-qPCR should be better at characterising the presence of viable cells as compared with quantitative cryptococcal culture. It suggests that VBNCs can be found in CSF and that antifungal treatment could induce this phenomenon. More work is needed to investigate VBNCs in human CSF under treatment.

In Botswana, the sensitivity and specificity of CryptoPS were 61·0% and 96·6%, respectively.[25] With this test, the C gattii species complex, including C bacillisporus, C deuterogattii, and C tetragattii species, was not detected in vitro.[33] Consistent with these findings, our results showed false negative results only in serotype B/C infections. The high prevalence of C gattii species complex infections in Botswana might explain the low sensitivity rate that was previously reported when using CryptoPS locally.[25]

One of the limitations of the study was that the qPCR assays were performed on frozen material and not in the centre that handled specimen culture, preventing work on fresh material for qPCR, whereas quantitative cryptococcal culture was performed in enrolling centres. We are not yet able to compare the identification performed with our qPCR assays with that obtained with whole-genome sequencing of the cultured isolates, but this work is ongoing.

In conclusion, we designed and validated three qPCR assays for the detection and quantification of Cryptococcus infections in the CSF. These assays have excellent correlation with the gold standard quantitative cryptococcal culture. qPCR will thus provide an easier fungal load monitoring tool and early information on subsequent outcome during HIV-associated cryptococcal meningitis in sub-Saharan Africa. As a diagnostic tool that can speciate and give fungal load, it will allow for new stratified management of patients. Our assays can be used to measure clearance more quickly and easily as an endpoint to assess novel antifungal regimens.

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Contributors

TM was involved in data collection, data analysis, literature search, verification of the underlying data, data interpretation, and drafting and writing of the manuscript. AS-L was involved in study design, data collection, and data analysis. KL, CK, TB-C, TL, MMoy, NY, DSL, HM, MMos, TSH, and JNJ were involved in participant enrolment and review of the manuscript. DSL was involved in the verification of the underlying data. JCH was involved in the manuscript review. OL was involved in the study design and manuscript review. AA was involved in study design, verification of the underlying data, data analysis, literature search, data interpretation, and writing and supervision of the project. All authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Declaration of interests

AA received honoraria for educational activities and webinars from Gilead Sciences and Pfizer and travel grants from Astellas and Gilead Sciences, outside the submitted work. All other authors declare no competing interests.

Data sharing

The anonymised data file can be shared, upon request made to the corresponding author (alexandre.alanio@pasteur.fr), via a link allowing the file to be downloaded though an institutional secured server.

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