Short-form paper:

Flow cytometry to assess CSF fungal burden in cryptococcal meningitis

(*Running title:* Flow cytometry counting of cryptococci)

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Abstract

Fungal burden in the cerebrospinal fluid is an important determinant of mortality in cryptococcal meningitis but its use to aid clinical decision-making is hampered by the time involved to perform quantitative cultures. Here we demonstrate the potential of flow cytometry as a novel and rapid technique to address this.

Text

Cryptococcal meningitis (CM) remains one of the commonest causes of meningitis in sub-Saharan Africa and a significant cause of death among persons with HIV-1 infection (1, 2). Cerebrospinal fluid (CSF) fungal burden is an important determinant of mortality but requires quantitative culture – a time consuming process taking several days limiting its usefulness as a clinical decision aid (3). Recent *in vitro* work using broth dilutions of *Cryptococcus neoformans* has demonstrated a very close association between the number of cryptococci counted using a flow cytometer and quantitative culture (4). This raises the possibility that flow cytometry might be a useful technique to rapidly assess fungal burden in patients with cryptococcal meningitis. However, no studies have examined this technique on *ex vivo* samples. We addressed this by performing flow cytometry counting of cryptococci in the CSF of patients with HIV-1-associated CM and compared these counts with measurement of fungal burden using quantitative CSF culture. This study formed part of a larger body of work primarily aimed at examining the CSF immune response in CM.

A prospective cohort study was conducted in Cape Town, South Africa. All participants provided written informed consent; surrogate consent was obtained from the next of kin for patients with impaired consciousness. Ethical approval was obtained from the University of Cape Town and Liverpool School of Tropical Medicine Research Ethics Committees. HIV-infected persons aged ≥18 years with a first episode of cryptococcal meningitis (diagnosed by antigen test or culture) were enrolled within 48 hours of diagnosis and lumbar puncture performed to measure CSF opening pressure. CSF fungal burden was assessed with quantitative culture (5) and cryptococcal antigen titre (CrAg® LFA, Immy, USA) (6) as previously described. The volume of remaining CSF was measured and the cells pelleted using centrifugation, this was incubated on ice with an amine viability dye (AQUA,

Invitrogen) and anti-CD45-PECy5.5 (Biolegend), then at room temperature with FACS lysing solution (BD Bioscience), protected from light at all times. Cells were fixed using 2% paraformaldehyde and analyzed within 24 hours on a BD LSR Fortessa Flow Cytometer using FACS Diva software (BD Biosciences). A forward scatter threshold of 5,000 was used to avoid including any debris in counting; the sample was acquired in its entirety to allow calculation of cell counts. Compensation was performed using species appropriate compensation beads (BD Biosciences; Invitrogen). Data was analysed using Flow Jo version 9.5.3 (Tree Star software) [**Figure 1**]. Cryptococci were defined as CSF cells negative for the pan-leukocyte marker CD45. Counts for the whole sample were divided by CSF volume to obtain *Cryptococcus* counts per mL CSF. Statistical analyses were performed using Stata version 12 (Stata Corp).

Sixty HIV-infected patients with cryptococcal meningitis were enrolled with a median CD4 count of 34 cells/μL. CSF samples were available for 58 participants, 36 of whom had not received any amphotericin B prior to enrolment. The median CSF volume collected for flow cytometry was 7 mL (interquartile range (IQR), 4.5-8). Median fungal burden was 4.74 $\log_{10}$ CFU/mL (IQR 3.5-5.75) measured by quantitative culture and 4.53 $\log_{10}$ *Cryptococcus* yeasts per mL (IQR 3.33-5.21) measured by flow cytometry. Median CrAg® LFA titre was 1:8000 (IQR 2000-16000).

Cryptococcal counts measured by flow cytometry were strongly correlated with both quantitative culture (Pearson's r=0.91, p<0.0001) [**Figure 2a**] and CrAg titre (Spearman rho=0.75, p<0.0001). Linear regression showed that quantitative culture result could accurately be predicted from flow cytometry counting ($\log_{10}$CFU/ml = 1.31 x $\log_{10}$ flow count – 1.28; R^2=0.82, p<0.0001). The agreement between flow cytometry counting and

quantitative culture was also assessed using a Bland-Altman plot [**Figure 2b**]. This showed good agreement between these two measurements with a mean difference of -0.1 $\log_{10}$ CFU/mL and only 6.9% (4/58) of values outside the 95% limits of agreement. These outlying values were mainly among participants with low fungal burdens (<500 CFU/mL) where flow cytometry counting produced values that were approximately 1-2 $\log_{10}$ CFU/mL higher. A similarly strong correlation and agreement between the two measurement techniques was also noted when analysis was restricted to the 36 participants who had not received any anti-fungal therapy prior to CSF sampling (Pearson's r=0.93, p<0.0001 [**Figure 2c**]; mean difference -0.30 $\log_{10}$ CFU/mL, with 11.1% (4/36) of values outside the limits of agreement) [**Figure 2d**].

These findings suggest that flow cytometry has the ability to provide a rapid and accurate measurement of fungal burden in persons with HIV-associated cryptococcal meningitis. If combined with a cryptococcal viability stain (as previously demonstrated *in vitro* (4)) flow cytometry could also be used to assess the response to treatment.

Due to the well-recognized toxicity of amphotericin B (7), there is considerable interest in short course regimens particularly in those patients with low fungal burden (8). Results from quantitative culture are not available in a timely enough manner to inform clinical decision making, but the rapidity of the result obtained from flow cytometric cryptococcal counting could potentially overcome this problem. This could allow for the reduction of both drug toxicity, cost and duration of hospitalization. Although this technique does require access to a flow cytometer, suitable machines are available in many laboratories in sub-Saharan Africa where they are used to measure CD4 count. In areas where they are not available, rapid assessment of fungal burden might be possible using quantitative microscopy (4).

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There were a number of limitations to this study. No cryptococcal specific stain was used to identify cryptococci, instead they were assumed to be any CD45 negative cell found in the CSF. Given that all participants had laboratory confirmed cryptococcal meningitis, a lysis buffer was used to ensure no erythrocytes remained in the CSF, and host leukocytes were excluded using the pan-leukocyte marker CD45, we feel this is a reasonable assumption and that our results are valid. However, to be a fully robust clinical assay an anti-cryptococcal stain would ideally be incorporated into the panel and the assay validated on CSF samples from patients who do not have CM. This would have the additional benefit of improving the accuracy of flow cytometric counting at low fungal burdens where debris or miscellaneous cells may have interfered with the counting process. In addition, we only examined the use of flow cytometric counting to assess fungal burden at baseline and did not assess changes on anti-fungal therapy. Future work should aim to incorporate a cryptococcal viability marker to address this issue as previously assessed *in vitro* (4).

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Conflict of Interest statement

None of the authors have any conflict of interest to declare.

Figure 1. CSF flow cytometry gating **(a1)** CSF cells (FSC-SCC) - poor definition of cell subsets due to cryptococci; **(a2)** CD45 and live-dead stain are used to separate cells; **(b)** FSC-SCC plot of cryptococci; **(c)** FSC-SCC plot CSF leukocytes; **(d)** FSC-SCC plot dead CD45+ cells.

Figure 2. (a) Scatterplot demonstrating association between flow cytometry counting and quantitative culture; **(b)** Bland-Altman plot showing good agreement between flow counting and quantitative culture (greyed area indicates the 95% limits of agreement); **(c)** Association between flow counting and quantitative culture among participants who had not received antifungal therapy before CSF sampling; **(d)** Bland-Altman plot showing agreement between the two techniques limited to participants who had not received antifungal therapy before CSF sampling (greyed area indicates the 95% limits of agreement).

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