Antifungal susceptibility does not correlate with fungal clearance or survival in AIDS-associated cryptococcal meningitis

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Key Words: Cryptococcal meningitis, susceptibility testing, HIV, outcome, mortality

Running title: Susceptibility testing and outcome in cryptococcal meningitis

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Abstract
We investigated the value of susceptibility testing in predicting response in AIDS-associated cryptococcal meningitis using clinical isolates from a randomized controlled trial of antifungal treatment (amphotericin monotherapy, amphotericin with flucytosine, or amphotericin with fluconazole). We found no correlation between antifungal susceptibility and either early or late survival, or fungal clearance.
Introduction

_Cryptococcus neoformans_ causes devastating meningitis and 15% of AIDS-related deaths globally. It is an environmental saprophyte acquired through inhalation; azole-resistance may reflect exposure to agricultural pesticides. WHO guidelines, available at [https://www.who.int/hiv/pub/guidelines/cryptococcal-disease/en/](https://www.who.int/hiv/pub/guidelines/cryptococcal-disease/en/), recommend induction therapy with amphotericin and flucytosine, which delivers improved cerebrospinal fluid (CSF) sterilisation and survival [1,2]. Flucytosine is unaffordable for countries with the greatest disease burden. Consequently, many patients receive inferior treatment with amphotericin, alone or with fluconazole [1].

Broth microdilution is the current standard method for antifungal susceptibility testing (AST) of yeasts, as outlined by the Clinical Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) [3]. There is substantial deviation from standardised methods in published literature, with varying media, incubation times and methods of end-point determination. The Sensititre YeastOne system (Thermo Fisher Scientific, UK), is a commercially available broth microdilution method for AST with good essential agreement with CLSI and EUCAST methods [3]. It has the advantages of ease of use and interpretation via a colorimetric binary output, and consistency through central manufacturing.

There is little consensus on the value of AST in cryptococcal meningitis; few studies demonstrate any association between susceptibility and outcome [4–6], although attempts using modified methods have been more successful [6]. The variety of methods, lack of susceptibility breakpoints for _C. neoformans_, heterogeneity of induction regimens and inconsistent assessment of baseline disease severity make these studies difficult to compare.
Previously, we reported the results of a randomised controlled trial (RCT) of induction therapy for AIDS-associated cryptococcal meningitis [1]. Here, we use isolates obtained at diagnosis in this study to determine the ability of AST to predict therapeutic response.

**Methods**

**Patient Population**

We enrolled 299 patients into an open-label RCT of antifungal therapy for AIDS-associated cryptococcal meningitis at a single center in Vietnam between 2005 and 2010. Detailed trial methodology has been described previously [1]. Patients received induction treatment with either amphotericin monotherapy (1mg/kg/day for 4 weeks), amphotericin combined with flucytosine (100mg/kg/day for 2 weeks) or amphotericin combined with fluconazole (400mg twice daily for 2 weeks), followed by consolidation with fluconazole monotherapy (400mg daily) until 10 weeks post-randomisation (see supplementary figure 1).

**Fungal isolates and susceptibility testing**

CSF quantitative fungal counts were determined as previously described [1]. *Cryptococcus* isolates were cultured from CSF at randomization and archived via a full plate sweep with storage on beads (Pro-Lab Diagnostics, UK) at -80°C. For AST, isolates were revived on Sabouraud plates, a single colony selected and purified by culture, and the susceptibility of this single isolate to amphotericin, fluconazole and flucytosine determined using Sensititre YeastOne as per the manufacturer’s instructions.

**Statistical analysis**

We analysed the joint effect of the relevant drug MICs for patients on combination therapy and the effect of amphotericin MIC for patients on monotherapy. Primary outcome was
survival until 70 days analysed using Cox regression. Secondary outcomes were survival until 14 days and 6 months, and CSF fungal decline over the first 14 days (estimated from longitudinal measurements during that period and a linear mixed-effects model). The Cox model was also analysed with adjustment for baseline fungal burden and Glasgow Coma Score (GCS), these factors being associated with worse outcome [1]. Data were included for all strains with a valid MIC result, and are presented for participants by trial arm in terms of the effect that a two-fold increase in MIC has on the outcome measure of interest. We also defined isolates as fully sensitive or not, using breakpoints from published literature and as suggested in CLSI guidelines; MIC $\leq 0.512\mu g/ml$ for amphotericin B, $\leq 4\mu g/ml$ for flucytosine, $\leq 8\mu g/ml$ for fluconazole at 72 hours [4]. All analyses were performed using R software version 2.13.1 (https://www.r-project.org/).

**Results**

Of 299 study participants, 23 were excluded (no viable baseline isolate n=9; inadequate growth by 72 hours n=12; missing purity plate data, n=2). Baseline characteristics of the primary analysis population are in supplementary table 1. Drug susceptibilities were similar between treatment arms (supplementary table 2); the range of susceptibilities is illustrated in supplementary figure 2.

**Primary outcomes (Patient survival)**

Table 1 shows the estimated effect on survival of decreasing antifungal susceptibility by 70 days post-randomisation, without adjustment for disease severity. There was no consistent trend in hazard ratios (HR) produced by the model. Due to the multiplicity of analyses, individual HR estimates and p-values should be interpreted with caution. The adjusted model produced similar results.
The Kaplan-Meier curves in supplementary figure 3 illustrate the estimated effect of antifungal susceptibility on time to death up to 6 months when patients’ isolates are categorised as either ‘fully sensitive’ or ‘non-susceptible’ (supplementary table 3). We found no evidence that this categorisation affected risk of death, including in an exploratory analysis of patients with high fungal loads (defined as \(>6\times10^6\) colony forming units/mL CSF).

**Secondary outcomes**

We found no evidence that antifungal susceptibility affected either the early (day 14) or late (6 month) hazard of death (Table 1). The adjusted model produced similar results for all outcome measures. We found no consistent effect of drug susceptibility on the rate of fungal clearance from CSF for any of the three drugs tested.

**Discussion**

The most effective induction regimen for cryptococcal meningitis, amphotericin combined with flucytosine, results in mortality rates of 15% to 40% [1,2]. Trial data suggests that amphotericin accelerates CSF fungal clearance, but amphotericin toxicity contributes to mortality when therapy continues for more than one week [2]. Given the high mortality rate, toxicity and cost of combination induction therapy, the ability to predict a patient’s response to antifungal therapy at diagnosis would enable optimisation of the limited therapeutic options available [6]. However, we found no evidence that AST, measured using Sensititre YeastOne, can help guide treatment choices in cryptococcal meningitis.
Some small studies report an association between *Cryptococcus* susceptibility and survival. Witt and colleagues found that fluconazole susceptibility was an independent predictor of treatment outcome (survival at 10 weeks with sterile CSF) in HIV-associated disease; however, they failed to demonstrate this association using the CLSI macrotiter method, used an amphotericin-free treatment regimen and did not adjust for baseline disease severity. A small, retrospective study by Lee (n=46) found an association between fluconazole susceptibility and survival, but none for amphotericin B or flucytosine. This study included a heterogeneous patient population managed with multiple treatment regimens, which were not adjusted for in the statistical analysis, potentially confounding results [6].

In contrast, larger studies, including ours, with more robust sampling and less selection bias by analysis of subsets of RCTs, fail to show an association between susceptibility and survival [4,5,7–9]. Our study’s strengths are its size, the randomized allocation of induction therapy (removing bias in treatment selection), standardisation of drug formulations, care delivery within a single institution and AST within a single laboratory. We found no evidence that AST of isolates at the point of diagnosis predicts mycological response or survival, even in patients with high fungal burdens. This was true for all 3 key antifungal drugs (amphotericin, flucytosine and fluconazole), for survival at both early (14 day) and late (70, 182 day) time-points, and following adjustment for baseline factors associated with severity, including fungal burden. We must conclude that AST has no utility in optimising therapy for patients with a first presentation of cryptococcal meningitis.

There are several possible explanations for the poor correlation between antifungal susceptibility of *C. neoformans* and therapeutic outcomes. These include significant differences between in vivo infection and in vitro AST environments. *C. neoformans* variably
expresses its phenotype in different models and culture systems, including virulence factors
(melanisation, polysaccharide capsule size, titan cell formation) which may influence
susceptibility. Secondly, host-drug interactions may play a role in clinical response;
amphotericin may have immunomodulatory effects promoting yeast clearance that cannot be
reflected in vitro, and may be variably expressed in AIDS patients [6].
A potential weakness of our study is that we tested only single purified isolates from our
patients. While the majority of immunosuppressed patients have infections from a single
strain of C. neoformans, multiple strain infections may occur in up to 18%, challenging the
concept of correlating outcome with the susceptibility of a single isolate [10]. A further
potential weakness is that we tested only baseline isolates; C. neoformans displays the
phenomenon of heteroresistance to azoles whereby a resistant sub-population can emerge
from within a predominantly susceptible single strain following azole exposure; this may
contribute to disease relapse [11,12].
In conclusion, we present robust data that AST of baseline isolates of C. neoformans in
AIDS-associated cryptococcal meningitis does not correlate with survival or mycological
clearance; it has no place in routine clinical use in first cases of cryptococcal meningitis.
However, several aspects warrant further investigation. Our study enrolled only severely
immunosuppressed patients, which may confound any effect of susceptibility on outcome;
similar data should be generated for immunocompetent patients. Furthermore, because of the
phenomenon of heteroresistance, AST may be more informative if measured following a few
days of treatment [11].

Funding
Supported by the Wellcome Trust [077078/Z/05/A and 089276/Z/09/Z] and British Infection
Acknowledgements

We thank the patients and staff of the Hospital for Tropical Diseases.

Potential conflicts of interest. MW is currently an employee of Roche Pharma. The contributions to this manuscript are unrelated to this position and were conducted during earlier employment or in his personal capacity only. J.D. reports grants from Wellcome Trust and the British Infection Society during the conduct of the study. L.O.C., D.V.A., T.T.H.C., N.V.V.C. and L.N.P.H. report no potential conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

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Table 1. The estimated effect, defined by hazard ratio (HR) and 95% confidence intervals, of a two-fold increase in the MICs estimated at 72 hours for amphotericin, fluconazole and flucytosine on survival outcomes at 14, 70 and 182 days post-randomisation for the primary analysis population and the effect on mycological outcome (the rate of decline of CSF fungal count, log10 Colony Forming Units/mL CSF/day).

|                      | Group 1 |                      | Group 2 |                      | Group 3 |                      |
|----------------------|---------|----------------------|---------|----------------------|---------|----------------------|
|                      | Amphotericin (n=92) | Amphotericin and Flucytosine (n=96) | Amphotericin and Fluconazole (n=88) |
| Death by day 14      | HR      | P value              | HR      | P value              | HR      | P value              |
|                      | 0.64    | 0.28                 | 0.86    | 0.75                 | 0.69    | 0.43                 |
|                      | (0.28, 1.44) |                      | (0.35, 2.13) |                      | (0.27, 1.75) |                      |
| Amphotericin         | -       | -                    | 0.70    | 0.34                 | -       | -                    |
| Flucytosine          | -       | -                    | (0.33, 1.47) |                      | -       | -                    |
| Flucytosine          | -       | -                    | -       | -                    | 1.23    | 0.54                 |
| Flucytosine          | -       | -                    | -       | -                    | (0.63, 2.43) |                      |
| Death by day 70      | HR      | P value              | HR      | P value              | HR      | P value              |
|                      | 0.94 (0.65, 1.86) | 0.83 (0.31, 1.09) | 0.58    | 0.58                 | 0.97    | 0.94                 |
| Amphotericin         | -       | -                    | 0.88    | (0.55, 1.39)         | -       | -                    |
| Flucytosine          | -       | -                    | 0.89    | -                    | 0.87    | 0.61                 |
| Flucytosine          | -       | -                    | (0.58, 1.39) |                      | (0.51, 1.49) |                      |
| Death by 6 months (day 182) | HR      | P value              | HR      | P value              | HR      | P value              |
|                      | 1.10 (0.65, 1.86) | 0.72 (0.34, 1.11) | 0.62    | 0.62                 | 1.28    | 0.42                 |
| Amphotericin         | -       | -                    | 0.89    | (0.58, 1.39)         | -       | -                    |
| Flucytosine          | -       | -                    | (0.80, 1.49) |                      | 0.86    | 0.51                 |
| Flucytosine          | -       | -                    | (0.54, 1.36) |                      | (0.54, 1.36) |                      |
| Change in CSF fungal decline in first 14 days (log10 CFU/mL of CSF per day) per two-fold increase in MIC | Effect estimate | P value | Effect estimate | P value | Effect estimate | P value |
| Amphotericin         | -0.01 (-0.07, 0.04) | 0.59 | 0.02 (-0.03, 0.07) | 0.40 | 0.00 (-0.04, 0.04) | 0.95 |
| Flucytosine          | -       | -                    | 1.10    | 0.63                 | -       | -                    |
| Flucytosine          | -       | -                    | (0.80, 1.49) |                      | 0.01    | 0.53                 |
| Flucytosine          | -       | -                    | (0.02, 0.04) |                      | -       | -                    |
When adjusted for baseline CSF log-quantitative fungal count, GCS below 15 and Cryptococcus genotype at recruitment the results for a two-fold increase in 72 hour MIC for amphotericin in group 2 were 0.55 (95% CI, 0.30-1.01), P value 0.053.

When adjusted as before, results for a two-fold increase in 72 hour MIC for amphotericin in group 2 were 0.58 (95% CI, 0.33-1.03), P value 0.06.
Supplementary Appendix

Methods for Susceptibility Testing

Minimum inhibitory concentrations (MICs) were estimated for all isolates after 72 hours incubation at 35°C. Isolates without growth at 35°C were incubated at 30°C, and MICs estimated as before. Candida krusei ATCC 6258 was used as a control strain.

Statistical methods

Quantitative fungal counts were log-transformed for all analyses. Prior to analysis, MICs below the limit of detection were replaced by \((\text{min}/2)\), where \(\text{min}\) is the lowest concentration of antifungal agent in the Sensititre YeastOne system; MICs above the limit of detection were replaced by \(2^*\text{max}\), where \(\text{max}\) is the highest concentration of antifungal agent.

Analyses were adjusted for randomised treatment assignment and tested for interaction between MICs and trial treatment arms. Analyses comparing the survival effect of ‘susceptible’ and ‘non-susceptible’ isolates in a Cox regression model were performed both with and without adjustment for baseline CSF log-quantitative fungal count, Glasgow Coma Scale score (GCS) below 15 at recruitment (previously identified as independent predictors of outcome), and AFLP cluster (VN1gamma versus not VN1gamma) [1,2].

Time to fungal clearance was estimated with a cause-specific Cox regression model adjusted for baseline CSF log-quantitative fungal count.

Results

CSF log-quantitative fungal count and GCS below 15 at recruitment produced similar results.
### Supplementary Table 1. Summary of baseline characteristics for the primary analysis population (n=276)

| Characteristic                              | Group 1 Amphotericin (n=92) | Group 2 Amphotericin and Flucytosine (n=96) | Group 3 Amphotericin and Fluconazole (n=88) |
|---------------------------------------------|------------------------------|---------------------------------------------|---------------------------------------------|
| Age – yr <sup>a</sup>                      |                              |                                             |                                             |
| Median                                      | 28                           | 28                                          | 27                                          |
| Interquartile range                         | 25, 31                       | 25, 33                                      | 24, 31                                      |
| Male sex – no. (%)                          | 76 (83)                      | 76 (79)                                     | 75 (85)                                     |
| Intravenous drug use – no./total no. (%)    | 47/84 (56)                   | 45/90 (50)                                  | 48/87 (55)                                  |
| Glasgow Coma Scale score <sup>b</sup>       |                              |                                             |                                             |
| Median                                      | 15                           | 15                                          | 15                                          |
| Interquartile range                         | 13, 15                       | 14, 15                                      | 15, 15                                      |
| CD4 count – cells/mm<sup>c</sup>            |                              |                                             |                                             |
| Median                                      | 18                           | 16                                          | 14                                          |
| Interquartile range                         | 8, 36                        | 9, 27                                       | 8, 41                                       |
| CSF opening pressure – cmH<sub>2</sub>O <sup>d</sup> |                              |                                             |                                             |
| Median                                      | 27                           | 32                                          | 24                                          |
| Interquartile range                         | 15, 40                       | 19, 40                                      | 16, 40                                      |
| CSF white cell count – cells/mL <sup>e</sup> |                              |                                             |                                             |
| Median                                      | 34                           | 26                                          | 25                                          |
| Interquartile range                         | 8, 86                        | 7, 63                                       | 7, 84                                       |
| CSF yeast count – log<sub>10</sub> CFU/mL <sup>f</sup> |                              |                                             |                                             |
| Median                                      | 5.95                         | 5.82                                        | 5.74                                        |
| Interquartile range                         | 5.60, 6.49                   | 4.70, 6.15                                  | 4.80, 6.23                                 |
| Weight – kg <sup>g</sup>                    |                              |                                             |                                             |
| Median                                      | 46                           | 48                                          | 48                                          |
| Interquartile range                         | 42, 50                       | 41, 50                                      | 45, 50                                      |

<sup>a</sup>Data were missing for 1 patient in group 3  
<sup>b</sup>Data were missing for 2 patients in group 1, 1 in group 2 and 1 in group 3  
<sup>c</sup>Data were missing for 23 patients in group 1, 24 in group 2, 23 in group 3  
<sup>d</sup>Data were missing for 16 patients in group 1, 19 in group 2, 17 in group 3  
<sup>e</sup>Data were missing for 9 patients in group 1, 10 in group 2, 10 in group 3  
<sup>f</sup>Data were missing for 19 patients in group 1, 19 in group 2, 19 in group 3  
<sup>g</sup>Data were missing for 2 patients in group 1, 2 in group 2 and 2 in group 3
Supplementary Table 2. Summary of susceptibility test outcomes by each trial treatment arm for the primary analysis population. MIC$_{50}$ and MIC$_{90}$; refer to the median and 90% quantile of isolates in the study respectively.

| 72 hour MIC (µg/mL) | Group 1 Amphotericin (n=92) | Group 2 Amphotericin and Flucytosine (n=96) | Group 3 Amphotericin and Fluconazole (n=88) |
|---------------------|-----------------------------|---------------------------------------------|---------------------------------------------|
| **Amphotericin**$^{a}$ |                             |                                             |                                             |
| MIC$_{50}$          | 0.512                       | 0.512                                       | 0.512                                       |
| MIC$_{90}$          | 1.024                       | 1.024                                       | 1.024                                       |
| Geometric mean      | 0.6089                      | 0.6688                                      | 0.6692                                      |
| Interquartile range | 0.256, 1.024                | 0.2560, 1.024                               | 0.256, 2.048                                |
| **Fluconazole**$^{b}$ |                             |                                             |                                             |
| MIC$_{50}$          | 8                           | 8                                           | 8                                           |
| MIC$_{90}$          | 16                          | 16                                          | 16                                          |
| Geometric mean      | 7.5846                      | 7.2687                                      | 7.1085                                      |
| Interquartile range | 0.125, 512                  | 1, 32                                       | 2, 32                                       |
| **Flucytosine**$^{c}$ |                             |                                             |                                             |
| MIC$_{50}$          | 8                           | 8                                           | 8                                           |
| MIC$_{90}$          | 16                          | 12                                          | 8                                           |
| Geometric mean      | 6.1224                      | 6.2586                                      | 6.6077                                      |
| Interquartile range | 0.96, 16                    | 2, 64                                       | 2, 128                                      |

$^{a}$Data were missing for 7 patients in group 1, 4 in group 2, 4 in group 3.

$^{b}$Data were missing for 1 patient in group 1, 2 in group 2, 1 in group 3 at 72 hours.

$^{c}$Data were missing for 1 patient in group 1, 1 in group 3 at 72 hours.

Supplementary Table 3

Classification of isolates as ‘fully sensitive’ or not as per CLSI suggestion$^{[3,4]}$

|                     | ‘Fully sensitive’ strains | Other |
|---------------------|--------------------------|-------|
|                     | N                        | N     |
| Amphotericin$^{a}$  | 173                      | 103   |
| Flucytosine$^{b}$   | 114                      | 162   |
| Fluconazole$^{c}$   | 213                      | 63    |

$^{a}$Fully sensitive = MIC ≤ 0.512µg/ml for amphotericin B

$^{b}$MIC ≤ 4µg/ml for flucytosine,

$^{c}$MIC ≤ 8µg/ml
Supplementary Figure 1. Study enrolment and treatment assignment for our RCT of induction therapy for AIDS-associated cryptococcal meningitis in Vietnam [1]
Supplementary Figure 2. Distribution of antifungal susceptibilities of all isolates for amphotericin, flucytosine and fluconazole. The diagonal shows histograms of log2-transformed MICs, the panels below the diagonal display scatterplots (with jittering to avoid over-plotting) and the panels above the diagonal show Pearson rank correlations.
**Supplementary Figure 3.** Kaplan-Meier curves illustrating the estimated effect of antifungal susceptibility on time to death when comparing ‘susceptible’ and ‘non-susceptible’ isolates of *C. neoformans* for amphotericin susceptibility among patients receiving amphotericin monotherapy induction; fluycytosine susceptibility among patients receiving flucytosine combination therapy induction; and fluconazole susceptibility across the primary analysis population and among patients receiving fluconazole combination therapy induction.
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