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Timed-kill curves for *Cryptococcus neoformans* isolated from patients with AIDS

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Infection with *Cryptococcus neoformans* is an increasing problem in immunocompromised patients, particularly those with acquired immune deficiency syndrome (AIDS). Amphotericin B and fluconazole are currently acceptable therapies for cryptococcal meningitis; however, their effects remain suboptimal and recurrence or treatment failure is still a problem. Antifungal susceptibility testing may be an important tool for guiding therapy, but for *C. neoformans*, a reliable method is still not available. This retrospective study evaluated minimal inhibitory concentration (MIC) for amphotericin B and fluconazole, and minimal fungicidal concentration (MFC) and timed-kill curves for amphotericin B against 16 clinical isolates of *C. neoformans* obtained from AIDS patients with cryptococcal meningitis. No correlation between clinical outcome and MIC was observed for amphotericin B. In selected cases, the MFC seemed to be a better predictor of outcome than MIC. In this study, amphotericin B timed-kill curves appeared to show a correlation with clinical outcome of the 16 patients with AIDS-associated cryptococcal meningitis. These *in vitro* tests must be further evaluated in prospective studies to confirm their potential usefulness for guiding cryptococcal meningitis therapy.

Keywords cryptococcal meningitis, *Cryptococcus neoformans*, susceptibility testing, timed-kill curves

Introduction

Infections caused by *Cryptococcus neoformans* are an increasing problem in immunocompromised patients, particularly those with acquired immune deficiency syndrome (AIDS), in whom this organism is the fourth most common cause of life-threatening infection. The incidence of cryptococcosis among AIDS patients has been estimated at 6–30% depending on the country and study. About 90% of AIDS patients infected with *C. neoformans* develop meningitis [1]. In the Hospital J. A. Fernández (Buenos Aires, Argentina), cryptococcal meningitis has been diagnosed in 6·9% of patients with AIDS, and is the defining illness in 61·2% of the cases [2]. Before the AIDS epidemic, the recommended therapy for cryptococcal meningitis was amphotericin B (0·3 mg kg day^{-1}) and flucytosine (150 mg kg day^{-1}) for 4 to 6 weeks [3]. Treatment of patients with AIDS and cryptococcal meningitis with low-dose amphotericin B (0·4 mg kg day^{-1}) or azole therapy has been associated with high mortality and low rates of cerebrospinal fluid sterilization. Recently, Van der Horst *et al.* [4] suggested as the treatment of choice an induction for 2 weeks with a higher dose of amphotericin B (0·7 mg kg day^{-1}) plus flucytosine (100 mg kg day^{-1}), followed by 8 weeks of therapy with

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oral fluconazole (400 mg day^{-1}). Given the high incidence of relapse after initial antifungal therapy, the current management of *C. neoformans* infections includes lifelong therapy with fluconazole. Despite this, recurrence or treatment failure among AIDS patients is still a problem.

The National Committee for Clinical Laboratory Standards (NCCLS) has developed a standardized antifungal susceptibility testing method for yeasts (M27-A) [5]. This method opened the door to correlate *in vitro* results with clinical outcome. While the M27-A procedure is reproducible, this does not guarantee detection of clinically relevant differences in susceptibility between isolates [6]. In addition, recent work has suggested that this method has only a limited ability to identify amphotericin B-resistant isolates of *Candida* and *Cryptococcus*. It has been recognized [7] that the growth of *C. neoformans* is suboptimal in RPMI 1640 and may not yield reliable results. Other authors have recommended alternative methodologies [8–12]. Thus, Ghannoum *et al.* [8] described a modified methodology for antifungal susceptibility testing of *C. neoformans* that involved the use of yeast nitrogen base (YNB) medium buffered to pH 7·0. This method produces a wider range of fluconazole minimal inhibitory concentrations (MICs) that seems to correlate with clinical outcome [13].

For some species of bacteria, studies have demonstrated that the bactericidal rate (timed-kill studies) may be a better determinant of clinical outcome than a simple numerical MIC or minimal bactericidal concentration (MBC). The estimation of the bactericidal activity becomes relatively more important in infections where host defenses do not play a major role, such as endocarditis, meningitis and osteomyelitis [14].

In this retrospective study, we have compared the results of antifungal susceptibility testing and the timed-kill curves for 16 isolates of *C. neoformans* with the clinical outcome in 16 AIDS patients with cryptococcal meningitis. Therefore, the purpose of this study was to explore if MICs, minimal fungicidal concentration (MFC) or timed-kill curves for amphotericin B showed a correlation with clinical outcome in such patients.

Materials and methods

Patients

Sixteen AIDS patients with a first episode of cryptococcal meningitis were included in this study. All of them had cryptococcal meningitis diagnosed by a cerebrospinal fluid (CSF)-positive culture for *C. neoformans*. The clinical records of all patients were retrospectively reviewed and the following variables were analyzed: age, sex, antifungal treatment and CD4 number. Mycology data collected were lumbar puncture findings, CSF cryptococcal antigen titer, Indian ink and results of culture.

Treatment

Patients with a first episode of AIDS-associated cryptococcal meningitis received an amphotericin B dose of 0·3 mg kg day^{-1} the first day and then 0·7 mg kg day^{-1} for the initial 2 weeks of therapy, followed by 8 weeks of treatment with fluconazole (400 mg day^{-1}). For long-term suppressive therapy, fluconazole (200 mg day^{-1}) was used.

Evaluation

Treatment was considered successful if the patient was alive and CSF was sterile at the end of 10 weeks of therapy.

Organisms

Sixteen strains were isolated from the CSF of the patients studied before treatment. Isolates were identified as *C. neoformans* based on the following parameters: morphology, assimilation and fermentation of carbon and nitrogen compounds [15].

In vitro *susceptibility testing*

A microbroth dilution method was used to determine MICs for amphotericin B and fluconazole. The culture medium employed was YNB medium (Difco Laboratories, Detroit, MI, USA) prepared following the manufacturer's instructions (10× stock). Then the medium was supplemented with 0·5% glucose [6] and buffered with sodium phosphate to pH 7·0 (YNBg) reaching a final 1× concentration.

Antifungal agents

Determination of minimal inhibitory concentration
The following antifungal agents were used in the study: amphotericin B (Bristol Myers Squibb, New Brunswick, NJ, USA) and fluconazole (Pfizer S.A., Buenos Aires, Argentina). The drugs were provided as standard powders of known potency. Stock solutions were prepared as follows: amphotericin B and fluconazole were dissolved in 100% dimethylsulfoxide (DMSO; Sigma Chemical Co, St Louis, MO, USA) at 0·6 and 6·0 mg ml^{-1}, respectively. The stock solutions were frozen at −20 °C. A series of 10 twofold dilutions was prepared from the

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stock solutions in DMSO from 0·02–10 μg ml^{-1} for amphotericin B and from 0·1–50 μg ml^{-1} for fluconazole. Each antifungal dilution was then diluted with 19 volumes of water, bringing their concentrations to three times the desired final concentrations. These solutions were pipetted in 50 μl volumes in each of the wells from column 1 to 10, sterile distilled water was dispensed in wells 11 and 12. Well 11 served as a sterility control and a blank for the spectrophotometric assays. Well 12 served as a growth control. In all experiments, flat-bottom microdilution plates (Nunclon 167008; Nunc, Naperville, IL, USA) were used. Microplates were stored at −20 °C for up to 6 months. The final concentrations of amphotericin B ranged 0·02–10 μg ml^{-1} and for fluconazole ranged 0·1–50 μg ml^{-1}.

The starting inoculum was obtained by suspending yeast cells in 5 ml of sterile 0·85% saline. The resulting yeast suspensions were vortexed for 15 s and the turbidity of *C. neoformans* was adjusted to 1 McFarland. Although the M27-A procedure suggests to adjust inocula to 0·5 McFarland, some authors observed that a higher initial inoculum for this microorganism led to improved growth [8]. YNBg medium was prepared to a concentration of 1·5 times the final concentration. The appropriate volume of the inoculum suspension was added (100 μl in 10 ml of YNBg medium). Then, 100 μl of the final inoculum suspension was added to each of the wells from column 1 through 10. This step produced a 1:3 dilution because each well contained 50 μl of antifungal agent. In well 11, 100 μl of YNBg medium without inoculum was dispensed (sterility control). In addition, 50 μl of inoculum suspension was added to well 12 (growth control). Following incubation at 35 °C for 48 and 72 h, the trays were shaken for 5 min at 150 rpm and turbidity was read at 405 nm with a Labsystem Multiskan RC microplate spectrophotometer (Labsystems Multiskan RC, Helsinki, Finland). MIC endpoints were defined as 80% of growth inhibition compared to the control [16].

Determination of minimal fungicidal concentration

For determining the amphotericin B MFC, 100 μl from each of the wells with growth inhibition were plated on yeast morphology agar (YM; Oxoid, London, UK). The plates were incubated at 35 °C. Colony counts were performed after 72 h. The MFC was defined as the concentration that killed ≥99·9% cfu ml^{-1}. The initial inoculum was between 1 and 5× 10^4 cfu ml^{-1} and the inoculum deposited in each of the wells between 0·3 and 20× 10^3 cfu ml^{-1}. Therefore, the MFC was defined as the lowest drug concentration that yielded three or fewer yeast colonies on the YM plate [17,18].

Timed-kill curves

Strains were grown in YNBg with agitation for 18 h at 35 °C. Initial inocula were adjusted to 1 McFarland (approximately 1× 10^6 cfu ml^{-1}). Two milliliters of the inocula were diluted in YNBg buffered with sodium phosphate to pH 7·0. This medium contained two different concentrations of amphotericin B (1 and 2 μg ml^{-1}). The final inoculum in the amphotericin B tubes was approximately 1× 10^5 cfu ml^{-1}. A control growth tube (20 ml of YNBg, pH 7·0) without amphotericin B was included in all experiments. The tubes were incubated at 35 °C. Colony counts were determined at 0, 6, 12, 24, 48 and 72 h. A 0·5 ml volume from each of the tubes was extracted and serial tenfold dilutions were performed. From each of these dilutions, 100 μl were plated on YM agar plates. After 72 h of incubation at 35 °C, colony counts were determined. The mean cfu ml^{-1} determined at time zero was considered the initial count. A killing of ≥99·9% of this inoculum was considered as the endpoint of the timed-kill curve, i.e. when ≤100 cfu ml^{-1} were enumerated on the YM agar colony count plate. A curve of inhibition plotting the cfu ml^{-1} against time was performed for each isolate. This technique allows estimation of the decrease in number of viable fungi over time.

Statistical methods

The relationship between CSF antigen titer and clinical outcome was analyzed by logistic regression. Multivariate analysis of variance (ANOVA) was used to compare the profiles obtained from the killing curves. Strains were divided into three groups: Group A ($n = 10$), isolates from patients who survived initial antifungal therapy; group B ($n = 3$), isolates from patients who died within the first 48 h of the initial amphotericin B therapy (total dose received <100 mg); and group C ($n = 3$), isolates from patients who died during the initial amphotericin B therapy (total dose received ≥500 mg).

Results

Clinical data

Patients and clinical data are shown in Table 1.

The use of logistic regression showed that there was no significant association between clinical outcome and CSF antigen titers. As shown in Table 1, there were patients from group A with very high CSF antigen titers that survived. Reciprocally, some of the dying patients had a similar CSF antigen titer to those surviving. In addition, the number of CD4 cells in both groups were similar, as all the patients had a CD4 count of ≤200 cells ml^{-1}.

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Table 1 Clinical and patient's data

Patient group	Patient number	Strain number	CD4 count	CSF antigen titer	AmB total dose (mg)	Outcome
Group A	1	955	<100	1/9192	1000	Survived
	2	1319	NA	1/128	1000	Survived
	3	1344	NA	1/128	1000	Survived
	4	1451	<50	1/128	1000	Survived
	5	1815	25	1/9192	1500	Survived
	6	1851	200	1/128	800	Survived
	7	1884	<100	1/4096	1000	Survived
	8	2032	100	1/4096	1000	Survived
	9	2086	<100	1/4096	1000	Survived
	10	2629	<50	1/128	1000	Survived
Group B	11	399	NA	1/9192	50	Died
	12	1036	NA	1/4096	80	Died
	13	2476	NA	1/1024	50	Died
Group C	14	947	50	1/4096	900	Died
	15	1130	75	1/1024	500	Died
	16	2672	100	1/128	600	Died

AmB, amphotericin B total dose received; NA, not available.
Other clinical data: age range, 25–37 years (average, 27·8 years); sex, 75% of the patients were male.

Unfortunately, group B CD4 numbers are not available due to the prompt fatal outcome. Ten of the 16 patients studied received amphotericin B and fluconazole therapy over 10 weeks. Strains isolated from these patients are listed in Table 1.

Three patients died at the beginning of amphotericin B therapy (1st and 2nd day, receiving a total dose of < 100 mg), the strains isolated were: 399, 1036 and 2476.

Three patients died during the administration of amphotericin B, despite receiving a total dose of ≥ 500 mg. Strains isolated were 947, 1130 and 2672.

MICs and MFCs

MICs of amphotericin B showed that all strains were inhibited by $\leq 1{\cdot}3\ \mu g\ ml^{-1}$ of amphotericin B. There were no differences higher than one or two dilutions between the endpoints at 48 and 72 h.

The MFCs of amphotericin B obtained for the 10 isolates of group A were all $\leq 2{\cdot}5\ \mu g\ ml^{-1}$. In group B, strain 399 (patient 11) had an MFC of $> 10\ \mu g\ ml^{-1}$, while the other two strains, 1036 and 2476 (patients 12 and 13, respectively) were killed by $2{\cdot}5\ \mu g\ ml^{-1}$. In group C, two strains (947 and 1130) had an MFC of $5{\cdot}0\ \mu g\ ml^{-1}$ and the third strain (2672) had an MFC of $2{\cdot}5\ \mu g\ ml^{-1}$.

All strains were inhibited by $\leq 6{\cdot}2\ \mu g\ ml^{-1}$ of fluconazole at 48 h. In view of these results, and taking into consideration the recently recommended break points of the NCCLS [7], it seems that these *C. neoformans* strains should be considered susceptible to fluconazole.

Timed-kill curves

The timed-kill curves of isolates of group A ($n = 10$) with an amphotericin B concentration of 1 or $2\ \mu g\ ml^{-1}$ were similar. All strains showed sustained inhibition of growth at 12 h (>2 log cfu ml^{-1}). After 48 h, all strains had a killing rate of $\geq 99{\cdot}9\%$ of the original cfu ml^{-1} (Fig. 1). The amphotericin B timed-kill curve at $2\ \mu g\ ml^{-1}$ showed a $\geq 99{\cdot}9\%$ killing rate at 6 h that was maintained throughout the experiment (72 h) (data not shown).

Group B comprised three patients that died within the first 48 h of amphotericin B treatment. Two strains from this group (1036 and 2476, patients 12 and 13, respectively; Table 1) had similar amphotericin B timed-kill

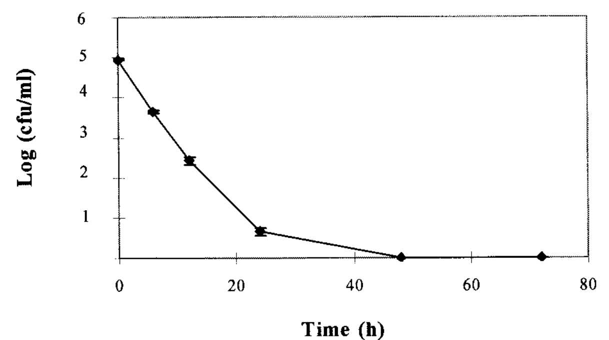

Fig. 1 Timed-kill curves of 10 isolates of *C. neoformans* from patients who responded to amphotericin B therapy (group A) against amphotericin B. The concentration of amphotericin B tested was $1\ \mu g\ ml^{-1}$. Every point represents the average of counts (cfu ml^{-1}) of 10 different strains at the indicated time. The bars are the standard errors of the average.

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curves at 1 μg ml^{-1} to those observed in group A. After 48 h, a ≥ 99·9% killing rate was detected. However, the third strain (399, patient 11; Table 1) showed a reduction of 2 log cfu ml^{-1} after 48 h of incubation. This isolate then recovered a normal growth rate and after 72 h, 1 × 10^5 cfu ml^{-1} were detected (Fig. 2).

The amphotericin B timed-kill curve at 2 μg ml^{-1} showed a similar inhibition and killing pattern for these three strains. The results were identical to those obtained with group A.

Group C comprised another three patients that died despite an amphotericin B total dose ≥ 500 mg. In all three cases, the amphotericin B timed-kill curve at 1 μg ml^{-1} showed a very slow initial inhibition of growth. After 12 or 24 h of incubation, a reduction of < 2 log cfu ml^{-1} was obtained. After this point, a normal pattern of growth was observed, and after 48 or 72 h, a count of 1 × 10^4 or 10^5 cfu ml^{-1}, respectively, was detected (Fig. 3). On the other hand, the amphotericin B timed-kill curves at 2 μg ml^{-1} were similar to those obtained with group A. All strains were killed after 6 h and did not revive after 72 h of incubation.

When the profiles of 1 μg ml^{-1} amphotericin B timed-kill curves were compared in an ANOVA test, the difference was significant ($P < 0{\cdot}002$). All timed-kill curves of isolates from Group B and C were tested in triplicate, showing similar results.

Discussion

Current general susceptibility testing for yeasts is not well suited for studies of *Cryptococcus* species. Most efforts towards developing a standardized reference method have involved *Candida* species as the main test organism [8].

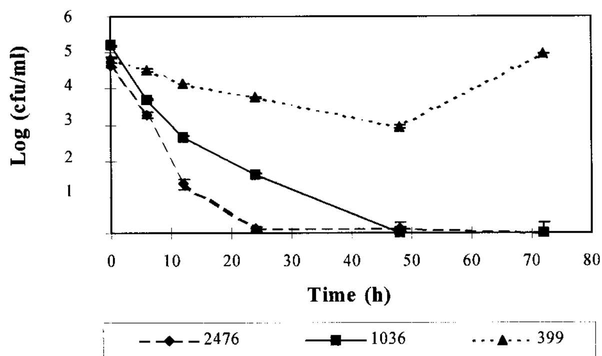

Fig. 2 Timed-kill curves of three *C. neoformans* strains isolated from patients who died at the beginning of amphotericin B therapy (group B). The concentration of amphotericin B tested was 1 μg ml^{-1}. Each timed-kill curve was performed in triplicate and the curve shown is the average of those triplicates. The bars are the standard errors of the average.

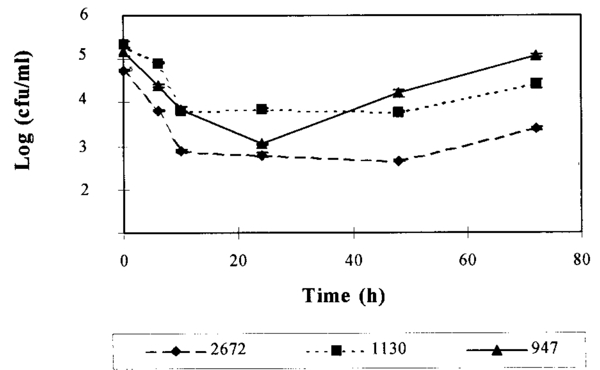

Fig. 3 Timed-kill curves of three *C. neoformans* strains isolated from patients who died during amphotericin B treatment (group C). The concentration of amphotericin B tested was 1 μg ml^{-1}. Each timed-kill curve was performed in triplicate and the curve shown is the average of those triplicates. The bars are the standard errors of the average.

Furthermore, M27-A, methodology [5] is not completely adequate for *C. neoformans*. Ghannoum *et al.* [8] developed a different method for *C. neoformans* antifungal susceptibility testing. Employing this methodology, Witt *et al.* [13] found a degree of correlation between fluconazole MICs and cryptococcal meningitis outcome. In that study [14], the authors identified as prognostic factors for a therapy failure, a positive blood or urine culture, a higher titer in serum or CSF of cryptococcal antigen and the fluconazole MIC. Emergence of fluconazole resistance in *C. neoformans* has also been detected in human immunodeficiency virus (HIV)-infected patients with primary fluconazole prophylaxis [19]. In recurrent cryptococcal meningitis, an increase in fluconazole MICs during relapse episodes has been reported [20]. The situation with amphotericin B is more complex as the M27-A susceptibility testing method appears to have difficulties identifying amphotericin B-resistant isolates [5].

In this work, we have determined the MICs, MFCs and timed-kill curves for 16 *C. neoformans* strains isolated from 16 AIDS patients with a first episode of cryptococcal meningitis.

Independently of patient's clinical outcome, the amphotericin B MICs of the 16 *C. neoformans* strains were very similar. All strains were inhibited by ≤ 1·3 μg ml^{-1}. Thus, in this study, MIC did not appear to have a prognostic value. Currently, for the correlation of amphotericin B and outcome, a method which readily identifies isolates known to be resistant is needed. However, there have been only two documented cases of amphotericin B-resistant *C. neoformans*, detected after prolonged amphotericin B therapy, in patients with AIDS

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[21,22]. In both studies, amphotericin B MICs of nine post-treatment isolates were higher than those obtained from pre-treatment strains.

In selected cases, the MFC appeared to have a predictive value. Three of our patients (patients 11, 14 and 15) died after infection with isolates for which the MFC was $\geq 5{\cdot}0\ \mu g\ ml^{-1}$. However, only two of these patients received a total amphotericin B dose > 500 mg. The third (patient 11) died within the first 48 h of amphotericin B treatment and no clear conclusion could be reached. On the other hand, 13 isolates had MFCs $\leq 2{\cdot}5\ \mu g\ ml^{-1}$. Three patients died and six survived (Table 1) with MFCs of $2{\cdot}5\ \mu g\ ml^{-1}$. Two out of three patients that died did not receive a therapeutic dose of amphotericin B, while the third received > 500 mg. It is presumed that unidentified host factors could have contributed to the patients' deaths. A possible preliminary conclusion is that MFC is a more important predictor of the efficacy of this fungicidal compound against *C. neoformans* than is MIC. An MFC $\geq 5{\cdot}0\ \mu g\ ml^{-1}$ may identify strains with a poor response to amphotericin B treatment.

The third determination performed (timed-kill curves) seemed to have a good prognostic value. Thus, 10 patients (group A; Table 1) that survived had *C. neoformans* that were inhibited and killed in 48 h with an amphotericin B concentration of 1 or 2 $\mu g\ ml^{-1}$ (Fig. 1). On the other hand, three patients that died despite a total amphotericin B dose ≥ 500 mg (group C; Table 1) had *C. neoformans* isolates that were initially inhibited, but after 12 or 24 h of incubation recovered the normal pattern of growth (Fig. 3). The analysis of group B (Table 1) is difficult because the patients died before 48 h of amphotericin B treatment. One of the strains (399; Fig. 3) showed a timed-kill curve similar to that seen in group C strains but the remainder resembled the group A trend. As is known with other systemic infections, indeterminate host factors may have influenced the clinical outcome for these patients.

The interpretation of our results is somewhat difficult. Some strains were not killed at an amphotericin B concentration of $1\ \mu g\ ml^{-1}$. However with $2\ \mu g\ ml^{-1}$, all isolates were killed at 6 h (data not shown). In our opinion, these strains cannot be considered resistant to amphotericin B. Analyzing the results of MFCs, some strains could be classified as tolerant [14]. However, for other strains also isolated from non-responding patients this was not the case (patient 16, strain 2672). The persistence of an initially small population that was not killed with $1\ \mu g\ ml^{-1}$ of amphotericin B was the only scenario observed. However, this population was not selected when a $2\ \mu g\ ml^{-1}$ of amphotericin B was used.

In previous studies, the amphotericin B resistance of *C. neoformans* strains seemed to be related to mutation [21,23] and was detected by MIC testing [24]. However, the possibility of other forms of resistance must be considered, such as tolerance or persistence. The employment of fungicidal tests allows the detection of these particular forms of resistance. This phenomenon in *C. neoformans* requires further study to establish a probable correlation between clinical response and treatment failure.

Although it is clear that there is no simple relationship between any available *in vitro* susceptibility tests and the *in vivo* response to antifungal therapy for *C. neoformans* in patients with AIDS, timed-kill curves may provide useful information in treatment surveillance. When an amphotericin B (at $1\ \mu g\ ml^{-1}$) timed-kill curve indicates that an isolate is inhibited ($< 2 \log cfu\ ml^{-1}$) but not killed at 48 h, other therapeutic options might be considered, e.g., adding flucytosine to amphotericin B treatment, administering an antifungal azole or using liposomal amphotericin B.

Although this is a small study, sufficient evidence is presented to show that the value of timed-kill curves as a potential tool for guiding the amphotericin B therapy of cryptococcal meningitis deserves further study. It does not seem a coincidence that three isolates recovered from patients with fatal outcome, despite the administration of 500 mg of amphotericin B, showed a timed-kill curve completely different that those of 10 strains isolated from patients who showed complete recovery. More studies are warranted.

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