Available online at www.sciencedirect.com
ScienceDirect
Journal of Microbiological Methods 72 (2008) 268–274
Journal of Microbiological Methods
www.elsevier.com/locate/jmicmeth

Candida albicans viability after exposure to amphotericin B: Assessment using metabolic assays and colony forming units

Prasanna D. Khot [a,1], Peter A. Suci [b,*], Bonnie J. Tyler [a]

[a] *Department of Chemical Engineering, University of Utah, Salt Lake City, UT 84112 United States*
[b] *Department of Microbiology and Center for Biofilm Engineering, 366 EPS, Montana State University, Bozeman, MT 59717-3980 United States*

Received 7 September 2007; received in revised form 11 December 2007; accepted 18 December 2007
Available online 23 December 2007

Abstract

Metabolic assays are a preferred method for evaluation of *Candida albicans* viability after exposure to antimicrobial agents in cases in which the culture is a complex mixture of yeast and filamentous forms. There is a lack of published data indicating the strength of the correlation between metabolic assays and viable cell numbers determined by a standard assay such as colony forming units (CFU). We developed a kinetic metabolic assay (KMA) for quantifying viable cells which was tested on yeast cells in both exponential and stationary phase using alamarBlue and XTT as metabolic indicators. The KMA enabled quantification of the viable population over a range of 10^1 to 10^7 cells that linearly correlated ($R^2 > 0.98$) with estimates made by enumeration of CFU regardless of the indicator or growth phase of the cells. Linear relationships were used to calibrate the KMA in terms of equivalent CFU. Viable cell populations were then determined after exposure to AmB. These results were compared with those obtained by direct enumeration of CFU. There were significant correlations between KMA-derived equivalent CFU and direct CFU estimates of viable cell populations for exponential-phase cells. However, the proportions of viable cells based on the KMA were consistently lower than those obtained directly by CFU. This trend was substantially more pronounced for stationary phase cells. These results show that even in the relatively simple case in which only the yeast form is present, the relationship between assessment by metabolic assays and CFU is perturbed by exposure to an antimicrobial and that, furthermore, growth phase alters the nature of the perturbation.

Keywords: XTT; AlamarBlue; Kinetic metabolic assay; *Candida albicans*; Antifungal susceptibility testing

1. Introduction

Candida albicans and other *Candida spp.* are the most common opportunistic fungal pathogens in humans (Snydman, 2003; Ruhnke, 2006). Antifungal susceptibility testing is a primary tool for guiding therapy (Rex et al., 2001; Patterson, 2002; Hospenthal et al., 2004; Pfaller, 2005). Metabolic assays that rely on reduction of a redox reactive dye (XTT or alamarBlue) have been widely accepted for antifungal susceptibility testing due to several advantages (Tellier et al., 1992; Pfaller et al., 1994; Hawser et al., 1998; Meletiadis et al., 2001; Yamaguchi et al., 2002; Espinel-Ingroff, 2006). They can be adapted for in situ testing, which captures some of the complexities of the pathogenic state of the organism and readily adapted for rapid and high throughput analysis (Ramage et al., 2001; Pettit et al., 2005; Antachopoulos et al., 2006). In addition, metabolic assays are preferred for systems where assessment based on colony forming units (CFU) or turbidity is intractable (Baillie and Douglas, 1999; Cuenca-Estrella and Rodriguez-Tudela, 2001; Ramage et al., 2001; Van Vianen et al., 2006). This is especially the case for susceptibility testing of fungal mycelia and biofilms that incorporate filamentous forms (Latge, 1999; Ramage et al., 2001; Lewis et al., 2005).

The standard for quantification of viable *C. albicans* is enumeration of CFU. Yet there are very few studies that have compared estimates of viable *C. albicans* obtained by a metabolic

* Corresponding author. Tel.: +1 406 994 7219; fax: +1 406 994 6098.
E-mail address: peter_s@erc.montana.edu (P.A. Suci).
[1] Present address: Fred Hutchinson Cancer Research Center, Program in Infectious Diseases, D3-100, Seattle, Washington, 98109-1024 United States.

0167-7012/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.mimet.2007.12.005

assay with direct CFU counts after exposure to an antifungal agent (Tellier et al., 1992; Ramage et al., 2001). Here, we describe a methodology for calibrating metabolic assays against standard CFU counts that is both sensitive and linear. We then provide a detailed assessment of how well our calibrated assay correlates with direct CFU counts for cells exposed to amphotericin B (AmB), a common antifungal agent of the polyene class.

2. Materials and methods

2.1. C. albicans strain and medium

C. albicans CA-1 is a clinical isolate obtained from the culture collection of Diane Brawner (Microbiology Department, Montana State University) that we have previously used for biofilm studies (Han et al., 1998; Suci and Tyler, 2003; Khot et al., 2006). The strain was stored at −80 °C. Planktonic cells were cultured in 2% YEPD medium (2% glucose, 1% bacto yeast extract, and 2% bacto peptone). The solid agar medium for the CFU assay was 1% glucose, 0.5% bacto yeast extract, 2% bacto agar, 0.1% ammonium sulfate dissolved in 20% tap water and 80% D.I. water.

2.2. Modified growth medium

In addition to 2% YEPD, the modified growth medium contained optimal concentrations of AmB-quenching reagents. The final concentrations of these reagents in the kinetic metabolic assays were 40 μM ergosterol (Alfa Aesar; catalog no. 57-87-4), 88 μM $MgCl_2$ (Sigma; catalog no. M4880) and 42.5 μM KCl (Sigma; catalog no. P5405) (Gale, 1974; Kerridge et al., 1976; Gale, 1986).

2.3. Planktonic cultures

Cultures were grown aerobically in 250-ml Erlenmeyer flasks containing 100 ml growth medium. The flasks were placed in a shaker incubator at 37 °C and 160 rpm for the desired period of growth. *C. albicans* grew as budding yeast under these conditions. Exponential phase cells were harvested after a 5-h growth period and stationary-phase cells at 96 h.

2.4. CFU assay

CFU were estimated for both AmB treated and untreated samples. 100 μl planktonic *C. albicans* cells in PBS were serially diluted in 2 ml cuvettes (catalog no. BTCUV, Biotrace Inc.). The serial dilution was 10-fold across each cuvette. The required numbers of serial dilutions per sample were judged based on trial and error. A volume of 100 μl from each serially diluted cuvette was spread as a separate lane on an agar plate. Each agar plate had a maximum of 4 lanes. Plates were incubated at 37 °C for 24 h. CFU were estimated from lanes of serial dilutions whose numbers fell in the range of 10 to 100 colonies per lane. The appropriate dilution factor was multiplied to estimate the final viable cell concentration for every sample.

2.5. Amphotericin B treatment

AmB was from Biosource International Inc. (Fungizone with 0.00205% sodium deoxycholate solubilizing agent). A standard broth dilution method was used to assess the AmB MIC of CA-1, with ATCC 24433 used as a reference strain (NCCLS., 1997). AmB treatment of planktonic cells was performed in 1.5-ml centrifuge tubes. The tubes contained a total working volume of 450 μl. AmB dissolved in 200 μl of 0.1 M PBS (pH 7.0) and cells resuspended in 250 μl of PBS adjusted to an optical density of 0.05 (A_{660}) were added to the 1.5-ml tube. The positive controls had no AmB, and the negative controls had no cells. In every experiment, a minimum of four negative controls were used. The AmB treatment period was 1 h. During AmB exposure, the 1.5-ml tubes were placed in a shaker incubator at 37 °C and 150 rpm. After AmB treatment, cells in 1.5-ml tubes were centrifuged for 5 min at 4000 ×*g*, the supernatant was decanted, and the pellet was resuspended in the same volume of fresh PBS buffer. A volume of 100 μl was used for every subsequent viability assay. The cells were transferred into wells of the 96-well plate (Corning Inc.; Costar 3370) system for the kinetic assays (XTT, alamarBlue and turbidity) and in the 2 ml cuvettes for the CFU assay.

2.6. Kinetic curves

Kinetic data of the reduction of metabolic indicators were recorded after AmB treatment. *C. albicans* were isolated by centrifugation from the solution with AmB and placed in fresh growth medium for the kinetic analysis. Kinetic data were independently generated with XTT (Sigma; catalog no. X4626) and alamarBlue (BioSource International; catalog no. DAL1100. Final concentration of menadione (catalog no. M5625) at 1 μM was used as an electron coupling agent with 0.05 mg/ml XTT. Each well had a total working volume of 230 μl. It contained 100 μl of cells in PBS from a sample, 25 μl metabolic dye, 75 μl of a PBS solution with AmB-quenching reagents (modified medium) or without them (unmodified medium) and, 30 μl 2% YEPD growth medium. The kinetic assay in the 96 well format was run for 24 h at 37 °C with continuous shaking in a Synergy-HT plate reader (Biotek Inc.).

Absorbance data was recorded at 492 nm and 660 nm for XTT and at 570 nm and 600 nm for the alamarBlue dye. Data were collected every 10 min for 24 h, generating 140 data points per sample. Each experiment resulted in an array of kinetic data corresponding to samples in the 96 well plate. Data were generated from at least three independent experiments. Kinetic data was exported from the KC4™ software (Biotek Inc.) into Microsoft Excel®. Matlab® codes specific to the calculation requirements of each metabolic indicator were used to automate data analysis. The percent reduction of the alamarBlue indicator was calculated as per the manufacturer's formula (product literature; BioSource International Inc.; catalog no. DAL1100). For the XTT indicator, final reduction of the dye was estimated by subtracting the absorbance value of 492 nm with the absorbance value at 660 nm. The value at 660 nm served as a reference for the XTT reduction and, also a measure of turbidity.

3. Data analysis

3.1. Calibration of the KMA

Equation 1 was used to model the exponential portions of the sigmoidal kinetic data curves. Eq. (2) is a linearized version of Eq. (1).

$$X_t = X_0 \exp(\mu t) \quad (1)$$

$$\ln(X_0) = \ln(X_t) - (\mu t) \quad (2)$$

X_t and X_0 are metabolic indicator concentrations at set threshold time (t) and at initial time ($t=0$) respectively. μ is a parameter that is related, but not necessarily identical to, the specific growth rate of the cells. Optimized fits of Eq. (1) to the exponential portions of the kinetic data curves (up to approximately 70% reduction) were used to obtain estimates of X_0 and μ, using a least squares algorithm (implemented by Matlab®'s curve fitting toolbox). The value of the constant from the fit was accepted only if the R^2 value exceeded 0.98. The kinetic parameters for each indicator were estimated for initial viable cells that ranged from approximately 10^1 to 10^7 CFU.

3.2. Calculation of percent viability

The viable fraction for KMA (based on either alamarBlue or XTT) or CFU assay was estimated as a percentage of its corresponding positive control (no AmB treatment). For the KMA, viability in terms of CFU was calculated by using the kinetic parameters estimated for each sample and the corresponding calibrations. Dose response curves contained percent viability plotted for increasing AmB concentrations. All results are presented as mean percent viability from three independent experiments ± standard deviation.

3.3. Correlation between KMA and CFU

To estimate the correlation in predicting viability between two independent assays, a Pearson's correlation coefficient (two-tailed) was estimated using Microsoft Excel® (Motulsky, 1995; Pettit et al., 2005). The 100% viability data points and all but one 0% viability data point (obtained for the lowest AmB concentration) were excluded from the analysis.

4. Results

4.1. Modeling of the KMA data for untreated cells.

For untreated cells linear relationships were obtained between CFU and viable cells predicted by the KMA on the basis of Eq. (2). The kinetic curves of metabolic reduction of XTT and alamarBlue followed a sigmoidal shape. The corresponding curves of turbidimetric changes were nearly identical, implying that the increase in reduced indicator was primarily due to cell proliferation (data not shown). Fig. 1 shows the kinetic curves for the alamarBlue assay for a wide range of the initial inoculum size (10 to 10^7 CFU). Similar curves were obtained with XTT. The curves proportionally shift to the right along the time axis as the size of the initial inoculum decreases. Qualitatively, this trend forms the basis for predicting viable cells in the sample (i.e., at time zero) from the KMA data curves. KMA data curves for both kinetic indicators were modeled. In order to obtain a quantitative metric, the exponential portions of the kinetic curves shown in Fig. 1, from 0% to approximately 70% reduction, were fit using Eq. 1, resulting in R^2 values that were >0.98 for all three assays (alamarBlue, XTT, turbidity).

Modeling the exponential portions of the KMA data curves using Eq. 1 resulted in optimization of two parameters (X_0 and μ) where X_0 is proportional to viable cells in at time zero. An example of the quality of the linear correlation between CFU at time zero and X_0 obtained from the fit of the data curve for

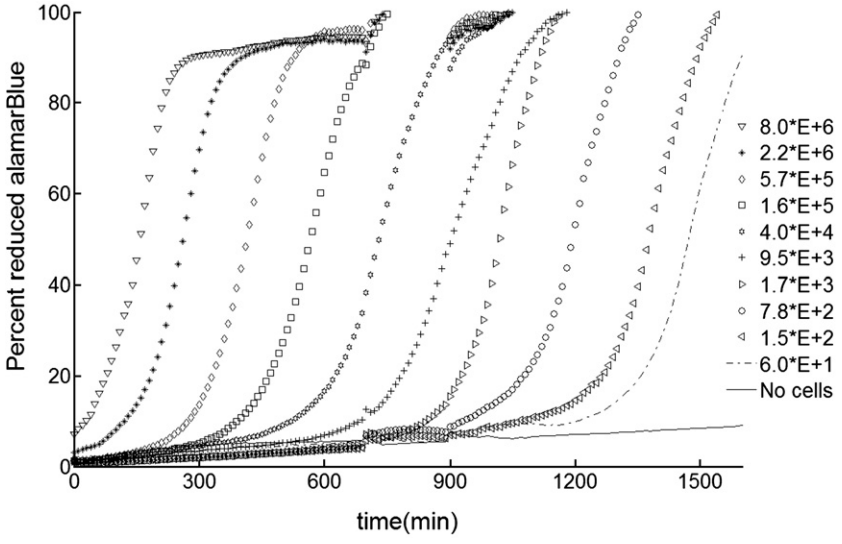

Fig. 1. Time course of metabolic reduction. Each curve represents an initial viable cell concentration ranging from 60 to 8×10^6 CFU.

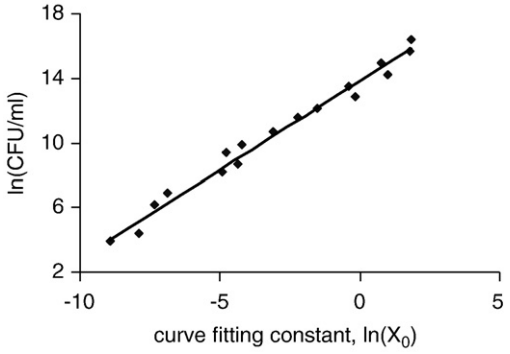

Fig. 2. Linear calibration for the kinetic metabolic assay using the alamarBlue indicator. The calibrations apply over a dynamic range of approximately six orders of magnitude ($10E^1$ to $10E^7$ CFU). Similar calibrations were obtained for all combinations of variables shown in Table 1.

reduction of the metabolic indicator is shown in Fig. 2. The curve fitting technique resulted in linear fits for all combinations of tested variables with R^2 values ranging from 0.91 to 0.98 and a dynamic range of six orders of magnitude (approximately 50 to 10^7 CFU) (Table 1).

4.2. Correlations between assays for cells exposed to AmB.

Cells were treated with AmB and KMA curves obtained and analyzed as described for untreated cells. The parameter X_0 was converted to viable cells in terms of equivalent CFU by using the linear relationships obtained for untreated cells (Table 1). A comparison was made of viable cell estimates (in terms of equivalent CFU) obtained by alamarBlue and XTT based KMAs and direct CFU counts. In addition, viable cell estimates obtained by alamarBlue and XTT based KMAs were compared to each other. Pearson's correlation coefficient was used to assess the quality of the correlations for each pair (Table 2). There was a good correlation between the metabolic assays based on reduction of the two indicators (alamarBlue and XTT) for all the tested conditions. In the case of the exponential-phase cells, the correlations between the CFU assay and metabolic assays based on both indicators (alamarBlue or XTT) were significant. For stationary-phase cells and the unmodified medium, the correlations between CFU and metabolic assays were poor. These correlations were strengthened when a modified medium was used, but the correlations were still lower in magnitude for stationary phase cells than the correlations obtained for the exponential-phase cells.

Table 1
Summary of KMA calibrations

Metabolic indicator	Growth medium	Inoculum growth phase	Curve fitting method [ln(CFU/ml)versus ln(X_0)]	
			Dynamic range (CFU/ml)	R^2*
alamarBlue	Unmodified	Stationary	8×10^6–60	0.97
alamarBlue	Unmodified	Exponential	1.28×10^7–50	0.98
alamarBlue	Modified	Stationary	8×10^6–60	0.98
alamarBlue	Modified	Exponential	1.28×10^7–50	0.93
XTT	Unmodified	Stationary	8×10^6–60	0.97
XTT	Unmodified	Exponential	3.1×10^6–50	0.98
XTT	Modified	Stationary	8×10^6–60	0.96
XTT	Modified	Exponential	1.28×10^7–220	0.91

*R^2 is the coefficient of determination for a linear fit.

Table 2
Correlation between assays

Growth phase-growth medium	AlamarBlue vs. XTT		AlamarBlue vs. CFU		XTT vs. CFU	
	r [1]	p [2]	r	p	R	P
Ex [3]–UM [4](df [5]=10)	0.800	0.01	0.848	0.01	0.904	0.01
Ex–M [6](df=13)	0.741	0.01	0.835	0.01	0.883	0.01
St [7]–UM (df=13)	0.752	0.01	0.279	*ns* [8]	0.327	*ns*
St–M (df=16)	0.679	0.01	0.555	0.02	0.571	0.02

A two-tailed (df=$n-2$) Pearson's correlation test was used.
[1] Pearson's correlation coefficient for comparing parametric data.
[2] level of significance for a two-tailed test.
[3] exponential phase.
[4] unmodified growth medium.
[5] degrees of freedom=$n-2$ for a two-tailed test, where n is the number of independent observations.
[6] modified growth medium.
[7] stationary phase.
[8] not statistically significant.

Compared to direct CFU, metabolic assays consistently underestimated the proportion of viable cells and this effect was more pronounced for stationary phase cells than for exponential phase cells (Fig. 3a and b). According to the metabolic assays, cells in both exponential and stationary phase assayed in the unmodified medium were completely eradicated at AmB concentrations greater than 0.88 μg/ml. In contrast, evaluation by direct CFU indicated that some cells survived AmB doses of 1.77 μg/ml and 14.12 μg/ml, for exponential and stationary phase cells, respectively. In addition, direct CFU was the most sensitive method for detecting differences in resistance between exponential- and stationary-phase cells. Quenching AmB activity using a modified medium improved the correspondence between estimates based on direct CFU and the metabolic assays (Fig. 3c and d) but relatively large discrepancies were still apparent, especially for stationary phase cells.

5. Discussion

Metabolic assays are typically used to assess the susceptibility of *C. albicans* to antifungal agents in cases in which the culture is a complex mixture of yeast and filamentous forms (Hawser, 1996; Chandra et al., 2001; Ramage et al., 2001; Khot et al., 2006; Perumal et al., 2007). The most commonly used metabolic assays measure reductive activity of dehydrogenases (Roehm et al., 1991; Gonzalez and Tarloff, 2001), whereas more conventional assays (CFU and turbidity) directly assess the ability of cells to proliferate. The good correspondence between break points obtained by metabolic and conventional turbidity assays (Example—NCCLS M27-A standard method) validates their application for MIC determinations (Pfaller and Barry, 1994; To et al., 1995; Davey et al., 1998; Espinel-Ingroff et al., 1999; Espinel-Ingroff, 2006). In principle, it should be possible to obtain a correspondence of similar convincing

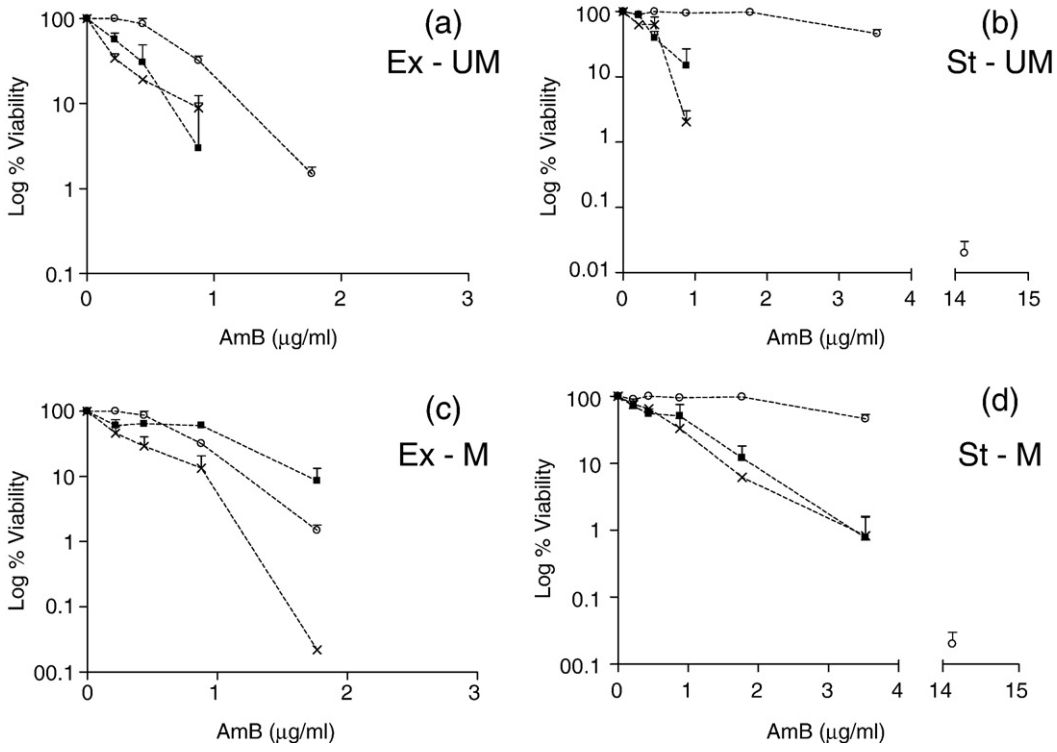

Fig. 3. Comparisons of KMA using XTT or alamarBlue with direct CFU. (■) alamarBlue; (×) XTT; and (o) CFU. (a) exponential phase cells, unmodified medium (Ex–UM); (b) stationary phase cells, unmodified medium (St–UM); (c) exponential phase cells, modified medium (Ex–M); (d) stationary phase cells, modified medium (St–M). Each data point is the average of 3 independent experiments ± standard deviation.

quality between estimates of viable cells based on metabolic assays and CFU that would validate their application for determining the fraction of cells that survive antimicrobial treatment. Our results confirm that it is possible to obtain an excellent linear correspondence between estimates of viable cells based on metabolic assays and CFU for untreated cells by measuring the kinetics of reduction of the metabolic indicator and fitting the data with an appropriate model. An additional benefit of this method is that the dynamic range is exceptionally broad (Table 1). Although the kinetics of reduction of a metabolic indicator have been used to follow the growth kinetics of cells (Kretschmar et al., 1996; Seligy and Rancourt, 1999), our method of determining viable cells from these kinetic data curves is novel (to the best of our knowledge) (Tyler and Khot, 2007).

Treatment with AmB reduced the quality of the linear correspondence between estimates of viable cells based on the KMA and CFU. In the case of stationary phase cells treated with AmB the correspondence was so radically altered that it became statistically insignificant. CFU and metabolic assays measure two independent, though related, parameters, i.e., ability of individual cells to proliferate and metabolic activity of the entire cell population. Our results suggest that the difference in estimates of viable cells based on CFU versus the metabolic assays originates from a perturbation of the growth rate of cells that are damaged by exposure to AmB, but nevertheless produce a CFU. Cells exposed to AmB produce colonies with a wide range of sizes whereas untreated cells produce colonies with a relatively tight size distribution. In most assessments based on metabolic assays, cells with slower growth and/or metabolic rates contribute proportionately less to the global estimate of viability. In contrast, cells that produce colonies contribute equally to the estimate of viability based on CFU regardless of the colony size. Thus it seems plausible that the relatively poor correspondence between the metabolic assays and direct CFU for stationary phase cells, compared to exponential phase cells, could have originated from the relatively slow recovery of stationary phase cells after exposure to AmB.

To assess if perturbation of growth rates in the KMA assays influenced their correlation with CFU, the medium used in the metabolic assays was modified to facilitate faster recovery after AmB treatment. AmB binds to ergosterol in the cell membrane of *C. albicans*. This alters the permeability of the membrane causing leakage of ions, eventually leading to cell death (Gale, 1986; White et al., 1998). Gale (1986) proposed a model of AmB action which essentially states that the cell wall lipids constitute a reservoir of AmB that feeds progressively into the membrane structure. This effect remains even when AmB is separated from the cells by centrifugation. This implies that AmB continues to cause damage of cells over a prolonged period. At higher concentrations of AmB, the progressive damage probably lasts longer. Previous work had suggested that adding optimal concentrations of extracellular ergosterol, $K^+$and Mg^{2+}ions considerably reduces or even arrests the damage AmB causes to *C. albicans* (Gale, 1974; Kerridge et al., 1976; Gale, 1986). This occurs either by antagonizing the AmB still present in the cell wall by ergosterol, and/or by neutralizing the ionic driving force

between the intracellular and extracellular ion concentrations by extracellular $K^+$and Mg^{2+}. Based on the analysis of the kinetic curves, modifying the growth medium by adding these reagents increased the growth (and metabolic reduction) rates of cells in the KMA (data not shown). An increase in growth rate resulted in a higher estimate of viable cells which correlated more significantly with CFU counts (Fig. 3).

If the kinetics of appearance of the metabolic indicator is exponential with no lag phase then the KMA should yield estimates of viable cells that are independent of the rate of reduction of the indicator, since the estimates are based on a projection of the kinetic data curve to time zero. However, if there is a lag phase in the kinetic data curves then the projected value for time zero will be an underestimate. Inspection of our data curves indicates that there is, in fact, a variable lag phase for treated cells. In addition, the lag phase appears to be proportionally more pronounced for samples treated with higher AmB concentrations. Therefore, a variable lag phase is at least partially responsible for the difference in estimates of the KMA and CFU.

In summary, for untreated cells an analysis of the KMA data curves was used to obtain parameters that were linearly related to direct CFU over a wide range of viable cells. Exposure of the cells to AmB perturbed this linear relationship, weakening the linear correspondence, especially for cells in stationary phase. The implication is that metabolic assays may lead to an underestimate of numbers of viable cells with slow growth rates. In some cases it is critical to sensitively detect viable slow growing cells. For example, slow growing cells may reseed a biofilm, contributing to the classical symptom of cycles of treatment followed by reappearance of the infection that is a hallmark of chronic biofilm infections (Costerton et al., 1999).

Acknowledgements

This study was supported by a grant from the National Institute of Dental and Craniofacial Research (DE13231-02) (B. J. Tyler), the National Institute of General Medical Sciences (1 R21 GM 079554-01A1) (P. A. Suci) and The University of Utah, Salt Lake City, UT.

We thank Dr. David Fredricks for critiquing this work.

References

Antachopoulos, C., Meletiadis, J., Roilides, E., Sein, T., Walsh, T.J., 2006. Rapid susceptibility testing of medically important zygomycetes by XTT assay. J. Clin. Microbiol. 442, 553–560.

Baillie, G.S., Douglas, L.J., 1999. Candida biofilms and their susceptibility to antifungal agents. Methods Enzymol. 310, 644–656.

Chandra, J., Mukherjee, P.K., Leidich, S.D., Faddoul, F.F., Hoyer, L.L., Douglas, L.J., et al., 2001. Antifungal resistance of Candidal biofilms formed on denture acrylic in vitro. J. Dent. Res. 803, 903–908.

Costerton, J.W., Stewart, P.S., Greenberg, E.P., 1999. Bacterial biofilms: a common cause of persistent infections. Science. 2845418, 1318–1322.

Cuenca-Estrella, M., Rodriguez-Tudela, J.L., 2001. Present status of the detection of antifungal resistance: the perspective from both sides of the ocean. Clin. Microbiol. Infect. 7 (Suppl 2), 46–53.

Davey, K.G., Szekely, A., Johnson, E.M., Warnock, D.W., 1998. Comparison of a new commercial colorimetric microdilution method with a standard method for in-vitro susceptibility testing of *Candida spp*. and *Cryptococcus neoformans*. J. Antimicrob. Chemother. 424, 439–444.

Espinel-Ingroff, A., 2006. Comparison of three commercial assays and a modified disk diffusion assay with two broth microdilution reference assays for testing zygomycetes, *Aspergillus spp.*, *Candida spp*., and *Cryptococcus neoformans* with posaconazole and amphotericin B. J. Clin. Microbiol. 4410, 3616–3622.

Espinel-Ingroff, A., Pfaller, M., Messer, S.A., Knapp, C.C., Killian, S., Norris, H.A., et al., 1999. Multicenter comparison of the sensititre YeastOne Colorimetric Antifungal Panel with the National Committee for Clinical Laboratory standards M27-A reference method for testing clinical isolates of common and emerging *Candida spp*., *Cryptococcus spp*., and other yeasts and yeast-like organisms. J. Clin. Microbiol. 373, 591–595.

Gale, E.F., 1974. The release of potassium ions from *Candida albicans* in the presence of polyene antibiotics. J. Gen. Microbiol. 802, 451–465.

Gale, E.F., 1986. Nature and development of phenotypic resistance to Amphotericin-B in *Candida albicans*. Adv. Microb. Physiol. 27, 277–320.

Gonzalez, R.J., Tarloff, J.B., 2001. Evaluation of hepatic subcellular fractions for Alamar blue and MTT reductase activity. Toxicol. In Vitro. 153, 257–259.

Han, Y.M., Morrison, R.P., Cutler, J.E., 1998. A vaccine and monoclonal antibodies that enhance mouse resistance to *Candida albicans* vaginal infection. Infect. Immun. 6612, 5771–5776.

Hawser, S., 1996. Comparisons of the susceptibilities of planktonic and adherent *Candida albicans* to antifungal agents: a modified XTT tetrazolium assay using synchronised C. albicans cells. J. Med. Vet. Mycol. 342, 149–152.

Hawser, S.P., Norris, H., Jessup, C.J., Ghannoum, M.A., 1998. Comparison of a 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) colorimetric method with the standardized National Committee for Clinical Laboratory Standards method of testing clinical yeast isolates for susceptibility to antifungal agents. J Clin Microbiol. 365, 1450–1452.

Hospenthal, D.R., Murray, C.K., Rinaldi, M.G., 2004. The role of antifungal susceptibility testing in the therapy of candidiasis. Diagn. Microbiol. Infect. Dis. 483, 153–160.

Kerridge, D., Koh, T.Y., Johnson, A.M., 1976. The interaction of amphotericin B methyl ester with protoplasts of *Candida albicans*. J. Gen. Microbiol. 961, 117–123.

Khot, P.D., Suci, P.A., Miller, R.L., Nelson, R.D., Tyler, B.J., 2006. A small subpopulation of blastospores in *Candida albicans* biofilms exhibit resistance to amphotericin B associated with differential regulation of ergosterol and beta-1,6-glucan pathway genes. Antimicrob. Agents Chemother. 5011, 3708–3716.

Kretschmar, M., Nichterlein, T., Nebe, C.T., Hof, H., Burger, K.J., 1996. Fungicidal effect of tyrothricin on *Candida albicans*. Mycoses 391–2, 45–50.

Latge, J.P., 1999. *Aspergillus fumigatus* and aspergillosis. Clin. Microbiol. Rev. 122, 310–350.

Lewis, R.E., Wiederhold, N.P., Klepser, M.E., 2005. In vitro pharmacodynamics of amphotericin B, itraconazole, and voriconazole against *Aspergillus*, *Fusarium*, and *Scedosporium spp*. Antimicrob. Agents Chemother. 493, 945–951.

Meletiadis, J., Mouton, J.W., Meis, J.F., Bouman, B.A., Donnelly, J.P., Verweij, P.E., 2001. Colorimetric assay for antifungal susceptibility testing of *Aspergillus* species. J. Clin. Microbiol. 399, 3402–3408.

Motulsky, H., 1995. Intuitive Biostatistics. Oxford University Press Inc., New York.

NCCLS., 1997. Reference method for broth dilution antifungal susceptibility testing of yeasts. NCCLS document M27-A. NCCLS, Wayne, Pa.

Patterson, T.F., 2002. Fungal susceptibility testing: where are we now? Transpl. Infect. Dis. 4 (Suppl 3), 38–45.

Perumal, P., Mekala, S., Chaffin, W.L., 2007. Antifungal drug resistance in *Candida albicans* biofilms: a role for cell density. Antimicrob. Agents Chemother. 51 (7), 2454–2463.

Pettit, R.K., Weber, C.A., Kean, M.J., Hoffmann, H., Pettit, G.R., Tan, R., et al., 2005. Microplate Alamar blue assay for *Staphylococcus epidermidis* biofilm susceptibility testing. Antimicrob. Agents Chemother. 497, 2612–2617.

Pfaller, M.A., Barry, A.L., 1994. Evaluation of a novel colorimetric broth microdilution method for antifungal susceptibility testing of yeast isolates. J Clin Microbiol. 328, 1992–1996.

Pfaller, M.A., 2005. Antifungal susceptibility testing methods. Curr. Drug Targets. 68, 929–943.

Pfaller, M.A., Grant, C., Morthland, V., Rhine-Chalberg, J., 1994. Comparative evaluation of alternative methods for broth dilution susceptibility testing of fluconazole against *Candida albicans*. J. Clin. Microbiol. 322, 506–509.

Ramage, G., vande Walle, K., Wickes, B.L., Lopez-Ribot, J.L., 2001. Standardized method for in vitro antifungal susceptibility testing of *Candida albicans* biofilms. Antimicrob. Agents Chemother. 459, 2475–2479.

Rex, J.H., Pfaller, M.A., Walsh, T.J., Chaturvedi, V., Espinel-Ingroff, A., Ghannoum, M.A., et al., 2001. Antifungal susceptibility testing: practical aspects and current challenges. Clin. Microbiol. Rev. 144, 643–658.

Roehm, N.W., Rodgers, G.H., Hatfield, S.M., Glasebrook, A.L., 1991. An improved colorimetric assay for cell proliferation and viability utilizing the tetrazolium salt XTT. J. Immunol. Methods. 1422, 257–265.

Ruhnke, M., 2006. Epidemiology of *Candida albicans* infections and role of non-*Candida-albicans* yeasts. Curr. Drug Targets 74, 495–504.

Seligy, V.V., Rancourt, J., 1999. Antibiotic MIC/MBC analysis of *Bacillus*-based commercial insecticides: use of bioreduction and DNA-based assays. J. Ind. Microbiol. Biotechnol. 226, 565–574.

Snydman, D.R., 2003. Shifting patterns in the epidemiology of nosocomial Candida infections. Chest 1235 (Suppl), 500S–503S.

Suci, P.A., Tyler, B.J., 2003. A method for discrimination of subpopulations of *Candida albicans* biofilm cells that exhibit relative levels of phenotypic resistance to chlorhexidine. J. Microbiol. Meth. 533, 313–325.

Tellier, R., Krajden, M., Grigoriew, G.A., Campbell, I., 1992. Innovative endpoint determination system for antifungal susceptibility testing of yeasts. Antimicrob. Agents Chemother. 368, 1619–1625.

To, W.K., Fothergill, A.W., Rinaldi, M.G., 1995. Comparative evaluation of macrodilution and alamar colorimetric microdilution broth methods for antifungal susceptibility testing of yeast isolates. J. Clin. Microbiol. 3310, 2660–2664.

Tyler, B.J. and Khot, P.D., 2007. Kinetic metabolic assay for antifungal susceptibility testing. Free Patents Online (Pending US patent publication # 20070238146). Application # 11/706,632.

Van Vianen, W., de Marie, S., ten Kate, M.T., Mathot, R.A., Bakker-Woudenberg, I.A., 2006. Caspofungin: antifungal activity in vitro, pharmacokinetics, and effects on fungal load and animal survival in neutropenic rats with invasive pulmonary aspergillosis. J. Antimicrob. Chemother. 574, 732–740.

White, T.C., Marr, K.A., Bowden, R.A., 1998. Clinical, cellular, and molecular factors that contribute to antifungal drug resistance. Clin. Microbiol. Rev. 112, 382–407.

Yamaguchi, H., Uchida, K., Nagino, K., Matsunaga, T., 2002. Usefulness of a colorimetric method for testing antifungal drug susceptibilities of *Aspergillus* species to voriconazole. J. Infect. Chemother. 84, 374–377.