

A simple and reproducible 96-well plate-based method for the formation of fungal biofilms and its application to antifungal susceptibility testing

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Published online 28 August 2008; doi:10.1038/nprot.2008.141

The incidence of fungal infections has increased significantly over the past decades. Very often these infections are associated with biofilm formation on implanted biomaterials and/or host surfaces. This has important clinical implications, as fungal biofilms display properties that are dramatically different from planktonic (free-living) populations, including increased resistance to antifungal agents. Here we describe a rapid and highly reproducible 96-well microtiter-based method for the formation of fungal biofilms, which is easily adaptable for antifungal susceptibility testing. This model is based on the ability of metabolically active sessile cells to reduce a tetrazolium salt (2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide) to water-soluble orange formazan compounds, the intensity of which can then be determined using a microtiter-plate reader. The entire procedure takes approximately 2 d to complete. This technique simplifies biofilm formation and quantification, making it more reliable and comparable among different laboratories, a necessary step toward the standardization of antifungal susceptibility testing of biofilms.

INTRODUCTION

Fungal infections caused by yeasts and moulds represent an escalating problem in health care, as advances in modern medicine prolong the lives of not only severely ill patients, including HIV-infected, cancer, transplant, surgical and ICU patients, but also newborn infants. Use of broad-spectrum antibiotics, neutropenia, parenteral nutrition, indwelling catheters, immunosuppression and disruption of mucosal barriers due to surgery, chemotherapy and radiotherapy represent the most important predisposing factors for these infections. *Candida* spp., *Aspergillus* spp. and *Cryptococcus neoformans* are among the most common etiologic agents of fungal infections^{1,2}. Fungi, being eukaryotic cells and more complex than bacteria, cause infections that are often difficult to diagnose and treat, and carry unacceptably high mortality rates³.

Most microbiology investigations have traditionally used free-living (planktonic) cells in pure culture, resulting in the common perception that microorganisms are independent unicellular life forms. However, in their natural ecosystems, most microbes exist as attached communities of cells within an organized biofilm and rarely as planktonic organisms⁴. Thus, a biofilm is defined as a surface-associated and highly structured community of microorganisms that are enclosed within a protective extracellular matrix. Microbial biofilms can form in nature but also inside a host, and in recent years, there has been an increased appreciation of the role that microbial biofilms play in human medicine: it is now estimated that about 65% of all human infections have a biofilm etiology⁵.

Many different groups of investigators have demonstrated that fungal biofilms show increased levels of resistance against various classes of antifungal drugs, most notably azoles and polyenes⁶. This resistance is likely multifactorial and, among other mechanisms, may be due to (i) high cellular density within the biofilm; (ii) the protective effect of the biofilm exopolymeric material; (iii) differential expression of genes linked to resistance, including those

encoding efflux pumps; and (iv) the presence of a subpopulation of 'persister' cells. However, newer antifungal agents, such as the echinocandins and liposomal formulations of amphotericin B, display increased efficacy against fungal biofilms^{7,8}. Of note, the commonly used Clinical and Laboratory Standards Institute (CLSI) broth microdilution techniques for antifungal susceptibility testing are based on the use of planktonic populations and will not enable prediction of the drugs' efficacy against fungal biofilms⁹. The increased levels of resistance typically associated with biofilms underscore the importance of developing standardized assays to test biofilm antifungal susceptibilities and to thereby systematically determine the effectiveness of different antifungal agents and regimens against fungal biofilms.

Traditionally, most models for the formation of microbial biofilms, including those formed by fungal species, are cumbersome, requiring expert handling, longer processing times and the use of specialized equipment not generally available in a regular microbiology laboratory. In the case of fungal biofilms, models used by different groups of investigators include the use of catheter disks, sheets and tubing from a variety of materials normally placed inside some type of sterile receptacle, glass and plastic slides, a perfused biofilm fermentor, microfermentors, cylindrical cellulose filters, acrylic strips and discs, germanium substratum, tissue culture flasks, syringes, modified Robbins devices, the Calgary biofilm device, the CDC reactor and so on, also including both biofilms formed under static and flow-through conditions^{10–21}. Perhaps with the exception of the Calgary biofilm device, most of these models are complex, technically demanding and generally not amenable to high-throughput screening, as relatively few equivalent biofilms can be produced at the same time⁶.

Here we describe a rapid and robust 96-well microtiter plate model for the formation of fungal biofilms. This technique involves

formation of multiple equivalent fungal biofilms on the bottom of wells of microtiter plates, coupled with a colorimetric method that measures the metabolic activities of cells within the biofilm based on the reduction of 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT). Upon processing by metabolically active cells, the XTT yields a water-soluble formazan-colored product that can be measured spectrophotometrically in a microtiter plate reader (Fig. 1). We (and others) have previously shown that the XTT-reduction assay shows excellent correlation between cellular density and metabolic activity, thus providing a semiquantitative measurement of biofilm formation^{22–26}. This colorimetric assay is noninvasive and non-destructive, requiring minimal post-processing of samples as compared with other alternative methods (such as viable cell counts) and has the additional advantage that, in contrast to other methods such as crystal violet staining and dry mass measurements^{25,27}, it correlates with cell viability, which is particularly useful for measuring the effects of drugs on biofilm cells. However, some caveats and limitations about the use of this reagent have been noted²⁷. For example, results using the XTT-colorimetric assay to compare biofilm-forming ability by different isolates need to be interpreted with caution, as it has been reported that different fungal species and even strains from the same species show marked differences in their ability to metabolize the XTT substrate²⁷. In addition, alterations in the metabolic states during the different phases of biofilm formation may lead to fluctuations in the ability of cells within the biofilms to metabolize this dye^{23,27}. Some more recent articles indicate that the use of other vital stains (SYTO9, propidium iodide), fluorogenic assays or bioluminescence may represent alternatives and have some practical advantages over the use of XTT^{23,24,28}.

This 96-well microtiter plate model for biofilm formation was originally developed for *Candida albicans* and other *Candida* spp.²⁵ but more recently has successfully been adapted for the formation of biofilms by other biofilm-forming fungal species of clinical interest, such as *Cryptococcus neoformans* and *Aspergillus fumigatus*^{29,30}. Overall, this microtiter plate-based model of biofilm formation offers a simple, flexible, relatively inexpensive, accurate and reproducible alternative for biofilm formation that is compatible with the widely available 96-well microplate platform. It can be used to examine multiple parameters and factors influencing biofilm formation, to estimate the biofilm-forming ability of multiple fungal isolates and mutant strains and, more importantly for the purpose of these studies, it can be easily adapted for antifungal susceptibility testing of fungal biofilms following the procedures described below^{25,31,32}.

MATERIALS

REAGENTS

- Sabouraud-dextrose agar (Becton Dickinson, cat. no. 211584) to prepare plates or slants for maintenance and short term storage of fungal isolates
- Yeast peptone dextrose (1% wt/vol yeast extract, 2% wt/vol peptone, 2% wt/vol dextrose, 1.5% wt/vol agar) (US Biological, cat. no. Y2076) liquid medium for propagation of fungal cultures
- RPMI 1640 without sodium bicarbonate supplemented with L-glutamine (Cellgro, cat. no. 50-020-PB) and buffered with 165 mM morpholinepropanesulfonic acid (Fisher, cat. no. BP308) to pH 7. From now on, this medium will be referred to simply as RPMI 1640.
- Dulbecco's modified Eagle's medium (DMEM; Cellgro, cat. no. 10-013-CV)
- Phosphate-buffered saline (PBS; 10 mM phosphate buffer, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4) (Sigma, cat. no. P4417)

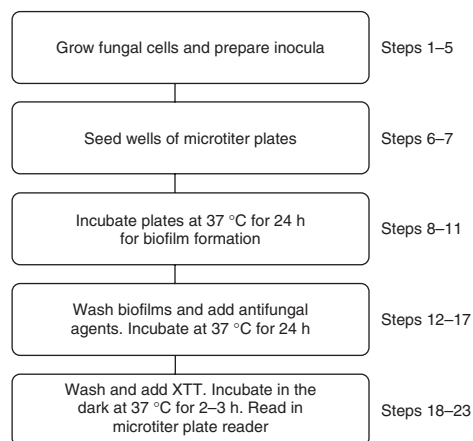


Figure 1 | A flow chart diagram summarizing the different steps of the procedure.

Experimental design

Fungal isolates. *Candida albicans* SC5314, *A. fumigatus* AF293 (NCPF 7367, CBS 101355) and *C. neoformans* H99 (serotype A) are all type strains that were used initially to optimize parameters of biofilm formation. Importantly, these are also the strains that were used for the corresponding genome sequencing projects of each fungal species and also represent parental strains used for the construction of a variety of mutants. These methods have been used to assess the biofilm-forming ability and antifungal susceptibility profiles of different clinical isolates of the three species and mutant strains, as well as representative isolates of other *Candida* spp. Stock cultures of these strains are typically prepared on Sabouraud dextrose agar plates or slopes and stored at 4 °C. For long-term storage, glycerol stocks and –70 °C are recommended.

Optimization of conditions. These procedures have already been optimized for each of the fungal species described here. Hence, we recommend readers to follow our suggested protocol, at least initially. On the basis of results obtained from their first experiments, readers can then try to optimize certain parameters as they apply to their own experimental design or particularities of their model (i.e., testing of clinical isolates, testing of different fungal species, testing biofilm-forming ability by mutant strains and testing different antifungal agents).

- Tween-20 (Bio-Rad, cat. no. 170-6531)
- Dimethyl sulfoxide (Sigma, cat. no. D8418)
- XTT sodium salt (Sigma, cat. no. X4251)
- Ringer's lactate (Hospira, cat. no. NDC0409-7953-09)
- Menadione (Sigma, cat. no. M5625) **! CAUTION** Menadione (2-methyl-1,4-naphthoquinone, also known as vitamin K₃) acts as an electron acceptor and can interfere with the electron transport chain in humans and as such is potentially toxic at high enough concentrations. It is extremely hazardous in case of skin contact, inhalation or ingestion. Always wear lab coat, gloves and mask when handling the powder.
- Acetone (Sigma, cat. no. 34850)
- Antifungal drugs: typically fluconazole (Pfizer Inc.), amphotericin B (Bristol-Myers Squibb) and caspofungin (Merck & Co. Inc.) as



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representatives of the three main classes of antifungal agents most frequently used in the clinics: azoles, polyenes and echinocandins, respectively.

EQUIPMENT

- Autoclave (Sanyo)
- Disposable plastic vacuum filter system, 500 ml (Corning, cat. no. 430769)
- Petri dishes (Fisher, cat. no. 08-757-12)
- 15-ml conical centrifuge tubes (Corning, cat. no. 430790)
- 50-ml conical centrifuge tubes (Corning, cat. no. 430828)
- Erlenmeyer flasks (Corning)
- Biological safety cabinet (Nuair)
- Vortex mixer (Fisher)
- Orbital shaker (New Brunswick)
- Centrifuge (Eppendorf)
- Hemacytometer (Hausser Scientific)
- Microscope (Fisher)
- 96-well microtiter plates: polystyrene, flat-bottomed, tissue culture-treated (Corning, cat. no. 3595)
- Parafilm
- Multichannel pipette and tips (Eppendorf)
- Incubator (Fisher)
- Inverted microscope (optional) (Fisher)
- Microtiter plate reader (Bio-Rad)

REAGENTS SETUP

Microbiological media If prepared from powder forms, after preparing the corresponding solutions in water following the manufacturer's instructions, microbiological media need to be autoclaved (yeast peptone dextrose) or filter-sterilized (RPMI 1640, DMEM). Once prepared, microbiological media can be stored at 4 °C for several months.

PROCEDURE

Growing the fungal cultures and preparation of inocula ● TIMING ~ 12–16 h (overnight)

1| For propagation of fungal cells, for *Candida* spp. and *C. neoformans*, flasks containing yeast peptone dextrose liquid medium (typically 20 ml of medium in a 150-ml flask) are inoculated with a loopful of cells from the stock cultures and incubated overnight in an orbital shaker (150–180 r.p.m.) at 30 °C. In the case of *A. fumigatus*, conidia are harvested from 3-day-old cultures on Sabouraud dextrose agar plates by flooding the surface of the plates with 5 ml of PBS containing 0.025% (vol/vol) Tween-20 and shaking gently. The conidial suspension is then recovered and dispensed into a 15-ml sterile tube. This conidial suspension can be retained for use for up to 2 weeks when stored at 4 °C.

! CAUTION Most fungal organisms to be tested here have the potential to cause infections in humans, particularly immunosuppressed individuals. Thus, adequate biosafety practices need to be followed. For *C. neoformans* and *Aspergillus* spp., all manipulations should be performed within a biological safety cabinet (in the case of *Aspergillus* spp., this is important also to prevent contamination of other laboratory surfaces). Work with *Candida* spp. can be performed on an open bench, but proper microbiological handling techniques to prevent contamination and universal precautions must be followed.

2| Harvest cells from the overnight-grown liquid cultures (for *Candida* spp. and *C. neoformans*) or from the conidial suspension (for *A. fumigatus*) by centrifugation (approximately 3,000*g* for 5 min at 4 °C), remove supernatant and wash twice in sterile PBS (by resuspending the pellet in approximately 20 ml of ice-cold buffer, vortexing vigorously, followed by centrifugation as mentioned above).

▲ CRITICAL STEP Because fungal cells tend to settle and/or aggregate, the cell suspensions must be vortexed vigorously after washings and before pipetting for the different manipulations used in this and successive steps.

3| Resuspend the final pellet of cells in approximately 20 ml of the appropriate medium that has been prewarmed to 37 °C: RPMI 1640 for *Candida* and *Aspergillus* and DMEM for *Cryptococcus*.

4| From the resulting cell suspension, prepare 1:100 and/or 1:1,000-fold dilutions in the same medium and count using a hemocytometer and a bright field microscope with a ×40 objective.

5| After counting, calculate the volumes needed to prepare a suspension of cells at a final density of 1.0×10^6 cells ml⁻¹ in RPMI 1640 for *Candida*, 1.0×10^5 cells ml⁻¹ in RPMI 1640 for *Aspergillus* or 1.0×10^7 cells ml⁻¹ in DMEM for *Cryptococcus*. The total volume needed will depend on the aggregate number of wells (or plates) that need to be seeded for biofilm formation for each isolate under investigation.

▲ CRITICAL STEP The fungal cell density of the initial inoculum is very important for correct biofilm development, as quorum-sensing mechanisms play an important role in biofilm formation. Cell densities that are too high or too low will likely

XTT XTT is prepared as a saturated solution at 0.5 g liter⁻¹ in sterile Ringer's lactate. Ringer's lactate can be substituted for PBS or any other physiological buffer, as these have been shown to give similar reproducible results. The XTT solution is light sensitive, so it should be covered with aluminum foil during preparation. The solution needs to be filter-sterilized using a 0.22- μ m pore size filter; as it is a saturated solution, the filtration step will leave yellow residues on the filter, but this does not constitute a problem. Once prepared and filter-sterilized, aliquot into 10 ml working volumes and store at -70 °C. We recommend wrapping the tubes containing this XTT solution in aluminum foil to prevent light penetration. We do not recommend storage of the XTT solution, even the frozen aliquots, for prolonged periods (longer than 1 year), as the activity of the reagent may decrease over time. Once the XTT is effectively metabolized by live fungal cells, this originally clear solution is transformed into an orange color. We have observed batch-to-batch variations between XTT solutions and even aliquots, but analysis and interpretation of results should not be problematic if proper intra-experimental controls are used. However, this variability makes it imperative to exercise caution when trying to compare absorbance readings from experiments performed on different days.

Menadione Prepare a 10 mM stock solution of menadione in 100% acetone. Aliquot into smaller volumes (about 50 μ l) and store at -70 °C.

Antifungal agents Antifungal agents are selected based on the testing required. The drugs are initially solubilized following manufacturer's instructions (most typically in dimethyl sulfoxide, but some antifungal agents are also soluble in water). If needed, concentrated stock solutions (i.e., 1 mg ml⁻¹) of the antifungal agents can be aliquoted into smaller volumes and stored at -70 °C until required.

result in poor biofilms. The cell densities for the inocula suggested here have been empirically optimized for experiments using 96-well microtiter plates for each different fungal species.

Biofilm formation ● TIMING ~ 24 h

6| Open as many 96-well microtiter plates as needed according to the experimental design (these plates are sterile and come individually packaged). We recommend performing a minimum of 2–4 replicates (entire rows of the microtiter plate(s)) for each combination of fungal isolate/antifungal agent to be tested.

7| From the standardized inoculum prepared in Step 5, pipette 100 μl (for *Candida*) or 200 μl (for *Aspergillus* and *C. neoformans*) into selected wells of the microtiter plate(s). Typically, wells in column 12 on each plate should remain unseeded, as these eight wells will act as negative background controls during subsequent analyses. Repeat as necessary for each different fungal isolate to be tested.

▲ **CRITICAL STEP** It is important that the seeding of the wells occurs promptly after preparation of the inocula.

▲ **CRITICAL STEP** If multiple rows are present in the same plate or the entire plate or if multiple plates are to be seeded with the same fungal isolate, the use of a multichannel pipette is strongly recommended for this and the successive steps.

8| When all the selected wells have been seeded, cover the entire microtiter plate with its original lid, seal with parafilm, place inside an incubator and incubate statically for 24 h at 37 °C. For *Candida* spp., *Aspergillus* spp. and *C. neoformans*, after 24- to 48-h incubation, biofilms should already display the complex three-dimensional architecture characteristic of multicellular communities, and the resulting biofilms can be used for antifungal susceptibility testing. However, after seeding the plates, the time of incubation for biofilm formation may be varied depending on the specific objectives of the study: for example, if the main objective of the study is examination of initial adherence, the incubation time can be reduced to 4 h; other investigators may want to study ‘fully mature’ biofilms after 72–96 h incubation.

! **CAUTION** Note that *A. fumigatus* biofilms with greater than 48 h growth may begin to sporulate, which could create post-processing problems and increase the possibilities for unwanted laboratory contamination.

9| After biofilm formation, aspirate the medium carefully as not to touch and disrupt the biofilm. The best way to do this is, using a multi-channel pipette, to angle the pipette tips toward the corners of the wells, thereby minimizing contact with the biofilm.

10| Using a multichannel pipette, wash plates three times in sterile PBS (200–300 μl per well) to remove planktonic and/or nonadherent cells that remain in the wells.

11| After each wash, the microtiter plates should be drained in an inverted position by blotting with paper towels to remove any residual PBS. Biofilms are now ready to be processed for antifungal susceptibility testing assays.

▲ **CRITICAL STEP** The washing procedures are critical, with the main emphasis on preserving biofilm integrity. Other methodologies can potentially be used, such as gently flicking the plates to discard the liquid contents, squirting buffer from a bottle, using an automated microtiter plate washer and so on. Typically, the fungal biofilms are strongly attached to the bottom of the wells of microtiter plates, and these washing procedures should not disrupt the preformed biofilms. In any case, at the end of the washing procedures, any well with clearly disrupted biofilm layer at the bottom (normally, this is visible by the naked eye) should be excluded from further subsequent calculations. This is one of the main reasons why we recommend performing sufficient replicates for each isolate/antifungal drug combination to be tested.

▲ **CRITICAL STEP** The ability to form multiple equivalent biofilms in a reproducible manner is a requirement for the success of these techniques. Thus, we recommend that when these protocols are followed for the very first time, and before proceeding to antifungal susceptibility testing, investigators corroborate that they have indeed been able to form multiple equivalent biofilms in the wells of the microtiter plate(s). At this point, after biofilm formation, the XTT/menadione reagent can be added and the resulting color read using a microtiter plate reader (see below, follow Steps 18–22; see also ANTICIPATED RESULTS). The same is true if assessment of biofilm-forming ability of fungal isolates (and not antifungal susceptibility testing) is the main objective of a particular set of experiments.

? **TROUBLESHOOTING**

Preparation of antifungal agents and antifungal challenge of preformed biofilms ● TIMING ~ 24 h

12| From the stock solution of each antifungal agent, a final ‘high’ working concentration is prepared in RPMI 1640 medium. Typical high concentrations are 1,024 $\mu\text{g ml}^{-1}$ for fluconazole and 16 $\mu\text{g ml}^{-1}$ for both amphotericin B and caspofungin. Other concentrations may be used for different agents.

▲ **CRITICAL STEP** If testing experimental and/or new agents with unknown activity against biofilms, we recommend to start with high concentrations of the drug, normally up to 100–1,000 times higher than one would use against planktonic populations.

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- 13|** Using a multichannel pipette, add 200 μl of the high working concentration of antifungal agent to the corresponding wells on column 1 of each microtiter plate containing fungal biofilms, being careful not to touch or otherwise disrupt the biofilms.
- 14|** Add 100 μl of RPMI 1640 to each well in columns 2–10. Add 100 μl of RPMI 1640 to wells in column 11; these will act as the positive control (biofilm not exposed to antifungal agent). Wells in column 12 remain empty as negative controls.
- 15|** Remove 100 μl of antifungal agent from the wells of column 1 and add to the adjacent wells in column 2 (already containing 100 μl of medium). The contents are then mixed by gently pipetting up and down to perform a serial doubling dilution, and the pipette tips are removed.
- 16|** Remove 100 μl of antifungal agent from the wells of column 2 and add to the adjacent wells in column 3 (already containing 100 μl of medium). Mix gently and repeat moving right until the wells of column 10, after which the final 100 μl volume from the wells of column 10 after mixing is discarded. You have just created serial doubling dilutions of your agent(s) of interest, from column 1 (most concentrated) to column 10 (least concentrated), with unchallenged biofilms in column 11 serving as positive controls.
- 17|** Cover the plates with their lids, seal with parafilm and incubate for 24 h at 37 $^{\circ}\text{C}$. Other incubation times can be used depending on the experimental design or particularities of the antifungal agent(s) being tested.

Postprocessing and measurement of metabolic activity after antifungal treatment ● TIMING ~2–3 h

18| Thaw as many tubes containing 10 ml of the XTT solution as required for the experimental design (one per plate). To each tube, add 1 μl of the stock solution of menadione to achieve a final menadione concentration of 1 μM . For uniformity, if multiple plates are processed at the same time, we recommend pooling all the resulting XTT/menadione tubes into a single solution in a clean sterile container.

19| Using a multichannel pipette, add 100 μl of the XTT/menadione solution to each well containing a prewashed biofilm as well as to negative control wells (for the measurement of background XTT-colorimetric levels).

20| Cover the plates in aluminum foil and incubate in the dark for 2–3 h at 37 $^{\circ}\text{C}$.

21| Uncover the plates. At this point, visual inspection of the plates will typically demonstrate a gradient of orange color. Using a multichannel pipette, remove 75–80 μl of the resulting colored supernatant from each well and transfer into the wells of a new microtiter plate.

? TROUBLESHOOTING

22| Read the plate (–) in a microtiter plate reader at 490 nm.

23| From the resulting colorimetric readings and after subtracting the corresponding values for negative controls (from wells in column 12 containing XTT only), calculate the sessile minimum inhibitory concentrations (SMICs) for each fungal isolate: SMIC50 and SMIC80 are the antifungal concentrations at which a 50% or 80% decrease in absorbance is detected in comparison with the control biofilms formed by the same fungal isolate in the absence of antifungal drug (in this case values for column 11). SMIC results are normally displayed in tabular form. Alternatively, results can be presented as a graph plotting colorimetric readings or percentage of inhibition (preferred) versus antifungal concentration (see ANTICIPATED RESULTS).

● TIMING

Steps 1–5, growing the fungal cultures and preparation of inocula: ~12–16 h (up to 3 d for *Aspergillus* spp.)

Steps 6–11, biofilm formation: ~24–48 h

Steps 12–17, preparation of antifungal agents and antifungal challenge of preformed biofilms: ~24 h

Steps 18–23, postprocessing and measurement of metabolic activity after antifungal treatment: ~2–3 h

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

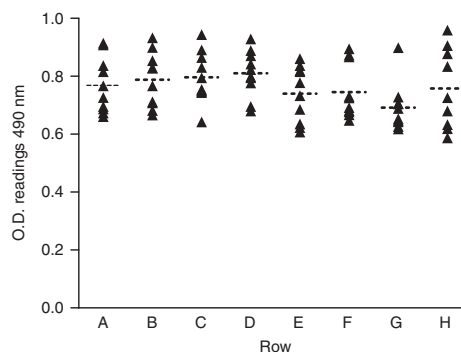


Figure 2 | Colorimetric readings (OD₄₉₀ values) from XTT-reduction assays of biofilms formed by *C. albicans* strain SC5314 in wells of microtiter plates. Values are for ten independent biofilms formed in each of eight different rows of the same 96-well microtiter plate. Dotted lines are mean values for each row.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
11	Lack of biofilm formation	Problems with counting the seeding inocula	Make sure that the dilutions and calculations are made appropriately
		Some fungal isolates do not form biofilm	These isolates should be considered as 'non-biofilm' formers Establish 'cut-off' values to identify nonbiofilm-forming isolates Consider using some other surfaces/materials for biofilm formation
All washing steps but particularly Step 11	Biofilms peel off from the bottom of wells	Aggressive washing	Be gentle during washings (follow recommendations) Most often, this will be visible by the naked eye. Do not use these wells for final calculation Make sure that enough replicates are performed to minimize problems
		Biofilms are too old (mature)	Disaggregation should be considered normal in the biofilm life cycle as biofilms reach maturity. Use younger biofilms
21	Biofilms visible by the naked eye but no orange color develops	Old XTT solution	Prepare a fresh solution of XTT
		Menadione is not added	Always remember to add menadione to XTT before adding to the wells
	Microbial contamination	Not maintaining sterility	This is a rare occurrence
		Poor handling	Always maintain proper microbiological handling techniques
	Storage of reagents	Minimize reopening of reagents. Discard any reagent with signs of contamination	

ANTICIPATED RESULTS

As emphasized before, it is important that multiple equivalent biofilms are formed in individual wells of the microtiter plates. **Figure 2** shows XTT-colorimetric readings (OD₄₉₀ values) for each of ten biofilms of *C. albicans* strain SC5314 formed in each of the eight different rows of the same 96-well microtiter plate (a total of 80 replicates). Results for the different rows were compared by one-way analysis of variance and using the Bartlett's test for homogeneity of variances and the Bonferroni's multiple comparison post-test. No statistically significant differences were noted when comparing all pairs of rows to each other ($P > 0.05$).

Table 2 shows typical results, expressed as SMIC50s and SMIC80s, of antifungal susceptibility testing for multiple *C. albicans* isolates against three of the most common antifungal agents used clinically. Note that typically biofilms are intrinsically resistant to fluconazole and most other azole derivatives. Amphotericin B is active against biofilms but normally at

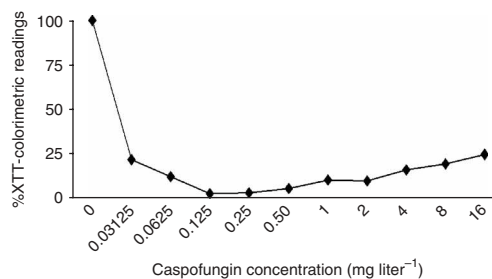
TABLE 2 | Results of antifungal susceptibility testing of biofilms formed by *C. albicans* type strain SC5314 and several *C. albicans* clinical isolates against fluconazole, amphotericin B and caspofungin.

Isolate	Fluconazole		Amphotericin B		Caspofungin	
	SMIC50	SMIC80	SMIC50	SMIC80	SMIC50	SMIC80
SC5314	> 1,024	> 1,024	0.5	1	0.03125	0.0625
No. 1	> 1,024	> 1,024	1	2	0.0625	0.0625
No. 2	1,024	> 1,024	0.5	0.5	0.125	0.25
No. 3	> 1,024	> 1,024	0.25	0.5	0.0625	0.0625
No. 4	1,024	> 1,024	0.5	1	0.03125	0.03125
No. 5	> 1,024	> 1,024	2	4	0.0625	0.125
No. 6	> 1,024	> 1,024	0.25	1	0.0625	0.0625
No. 7	> 1,024	> 1,024	0.5	1	0.0625	0.0625

Values are in mg liter⁻¹.



Figure 3 | Activity of different caspofungin concentrations against preformed biofilms of *C. albicans* SC5314. Values are expressed as average percentage of colorimetric readings for XTT-reduction assays as compared with control wells. Note that, as previously described, caspofungin loses efficacy at nonphysiological high concentrations. This has been referred to as the ‘paradoxical’ or ‘Eagle’ effect.



concentrations considered to be high, due to the intrinsic toxicity associated with this polyene antibiotic. Caspofungin, a representative of the echinocandin class of new antifungal agents, shows excellent *in vitro* activity against biofilms formed by most *C. albicans* isolates. **Figure 3** shows the activity of caspofungin at different concentrations against *C. albicans* biofilms, expressed as percentage of colorimetric readings for XTT-reduction assays as compared with control wells.

ACKNOWLEDGMENTS Work in the laboratory is funded by Grant number 5R21DE017294 from the National Institute of Dental & Craniofacial Research (to J.L.L.-R.). The content is solely our responsibility and does not necessarily represent the official views of the NIDCR, the NIAID or the NIH. A.R.T. was supported by the McNair Scholars Program at St Mary’s University funded by the US Department of Education.

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