Video Article

A 96 Well Microtiter Plate-based Method for Monitoring Formation and Antifungal Susceptibility Testing of *Candida albicans* Biofilms

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Abstract

Candida albicans remains the most frequent cause of fungal infections in an expanding population of compromised patients and candidiasis is now the third most common infection in US hospitals. Different manifestations of candidiasis are associated with biofilm formation, both on host tissues and/or medical devices (i.e. catheters). Biofilm formation carries negative clinical implications, as cells within the biofilms are protected from host immune responses and from the action of antifungals. We have developed a simple, fast and robust *in vitro* model for the formation of *C. albicans* biofilms using 96 well microtiter-plates, which can also be used for biofilm antifungal susceptibility testing. The readout of this assay is colorimetric, based on the reduction of XTT (a tetrazolium salt) by metabolically active fungal biofilm cells. A typical experiment takes approximately 24 h for biofilm formation, with an additional 24 h for antifungal susceptibility testing. Because of its simplicity and the use of commonly available laboratory materials and equipment, this technique democratizes biofilm research and represents an important step towards the standardization of antifungal susceptibility testing of fungal biofilms.

Video Link

The video component of this article can be found at https://www.jove.com/video/2287/

Protocol

1. Preparation of *C. albicans*

C. albicans is a Risk Group 1/BSL1 microorganism. Always remember to use good aseptic/sterile techniques for work with this microorganism and follow institutional procedures for proper disposal of biohazard materials.

1. Prepare an overnight culture of *C. albicans* in YPD (Yeast Peptone Dextrose) liquid medium by inoculating a single colony of *C. albicans* into 25 mL of YPD.
2. Incubate culture in an orbital shaker (about 180 rpm) at 30 °C overnight. Most *C. albicans* strains will grow as yeast cells under these conditions.
3. Centrifuge the overnight cultures (about 3,000 rpm, 5-10 minutes), wash cells twice with sterile PBS, and resuspend the final pellet in about 20-25 mL of RPMI 1640 medium buffered with 165 mM morpholinepropanesulfonic acid to pH 7.0 and pre-warmed at 37 °C (from now on this medium is referred to as simply "RPMI 1640").
4. Count cells using an hemacytometer. After counting, prepare a suspension of cells at a final density of 1.0 x 10^6 cells/ mL in RPMI 1640. Note: Because cells have a tendency to aggregate, it is important to vortex vigorously between washings and before pipetting.

2. Setting Up the 96-well Microtiter Plate for the Formation of the Biofilm

1. Using a multichannel pipette add 100 µL of *C. albicans* suspension into selected wells of the 96-well microtiter plate(-s). Do not add cells to wells in column 12, as these will serve as negative controls.
2. Cover the entire microtiter plate with its original lid, seal with parafilm, place inside an incubator and incubate for 24 h at 37 °C. The length of incubation can be adapted to the specific experimental design. For example, it is possible to examine the kinetics of biofilm formation over a period of 24 - 48 hours by seeding multiple plates and processing each plate at different time points (i.e. 2, 4, 8, 12, 24 and 48 h)
3. After biofilm formation, using a multichannel pipette aspirate the medium carefully as not to touch and disrupt the biofilms that have formed in each of the wells.
4. Using a multichannel pipette wash plates three times in sterile PBS (200 µL per well). Alternatively, use an automated microtiter plate washer. Between washes, and particularly after the last wash, drain the plates in an inverted position by blotting with paper towels to remove any

residual PBS. At this point biofilms formed on the bottom of the wells should be clearly visible even by the naked eye and can also be visualized using an inverted microscope (Figure 1). Biofilms are now ready to be processed for antifungal susceptibility testing assays. (If the main purpose of the experiment is to assess the extent of biofilm formation, the plates are ready to be processed using the colorimetric method. For this, the XTT/menadione reagent (see Step 3 below) can be added and the resulting color read using a microtiter plate reader).

3. Antifungal Susceptibility Testing of Biofilms

1. From stock solutions or powder, prepare a working solution in RPMI 1640 medium of each antifungal to be tested. Typical high concentrations are 1,024 µg/ mL for fluconazole, and 16 µg/ mL for both amphotericin B and caspofungin. Other concentrations may be used for different agents.
2. Using a multichannel pipette, add 200 µL of the high working concentration of antifungal to the corresponding wells in column 1 of each microtiter plate containing fungal biofilms.
3. Add 100 µL of RPMI 1640 to each well in columns 2 to 10.
4. Add 100 µL of RPMI 1640 to wells in column 11.
5. 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).
6. Mix the contents well by pipetting up and down to perform a serial doubling dilution, and remove the tips.
7. 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. In this way, a series of doubling dilutions of your agent(-s) of interest have been created; from most concentrated in wells of column 1 to least concentrated in wells of column 10. Unchallenged biofilms in column 11 will serve as positive controls, and empty wells in column 12 will serve as negative controls.
8. Cover the plates with their lids, seal with parafilm and incubate for 24 -48 h at 37 °C.
9. After the incubation period wash the plates as in Step 2.4 before (3 x PBS).
10. Using a multichannel pipette add 100 µL of XTT/menadione solution to each well containing a pre-washed biofilm as well as to negative control wells for the measurement of background XTT-colorimetric levels.
    1. The XTT is prepared as a saturated solution at 0.5 g/L in sterile Ringer's lactate, PBS or saline, which needs to be filter-sterilized using a 0.22 µm-pore size filter. The XTT solution is light sensitive, so it should be covered with aluminum foil during preparation. Once prepared and filter-sterilized, aliquot into 10 mL working volumes, and store at -70 °C. Protect the tubes from light using aluminum foil. Thaw only as many tubes as needed for a particular experiment just prior to use.
    2. Menadione is prepared as a 10 mM stock solution in 100% acetone, aliquoted into smaller volumes (about 50 µL) and stored at -70°C. Prepare the XTT/menadione solution just prior to use, by adding 1 µL of the stock solution of menadione to a tube containing 10 mL of the thawed XTT solution.
11. Cover the plates in aluminum foil and incubate in the dark for 2 h at 37 °C.
12. Uncover the plates. Using a multichannel pipette remove 80 µL of the resulting colored supernatant from each well and transfer into the corresponding wells of a new microtiter plate.
13. Read the plate(-s) in a microtiter plate reader at 490 nm.
14. Calculate the sessile minimum inhibitory concentrations SMIC50 and SMIC80, which are the antifungal concentrations at which a 50% or 80% decrease in colorimetric readings are detected in comparison to the control biofilms formed in the absence of antifungal drug (in this case values for column 11, remember also to subtract values from negative controls from wells in column 12 containing XTT only). SMIC results can be presented as a Table (i.e. multiple isolates against multiple antifungals) or alternatively, results for each individual fungal isolate against each antifungal can be presented as a graph by plotting percent inhibition versus antifungal concentration.

Example: After subtracting the values in the negative control, the average O.D. of control biofilms formed in column 11 is 1.32. The SMIC50 is the lowest antifungal concentration leading to > 50% reduction in colorimetric readings, in this case less than 1.32 x 50/100 = 0.66. Likewise, the SMIC80 is the lowest antifungal concentration leading to > 80% reduction in colorimetric readings, in this case less than 1.32 x 20/100 = 0.264.

4. Representative Results

Figure 1 shows a microphotograph of a *C. albicans* biofilm formed on the bottom of a well in a 96 well microtiter plate taken using an inverted microscope. Figure 2 shows XTT-colorimetric readings (OD_{490} values) for each of 11 biofilms of a *C. albicans* wild type strain formed in each of the 8 different rows of the same 96 well microtiter plate. Figure 3 shows the activity of amphotericin B at different concentrations against *C. albicans* biofilms; arrows indicate SMIC50 and SMIC80 values.

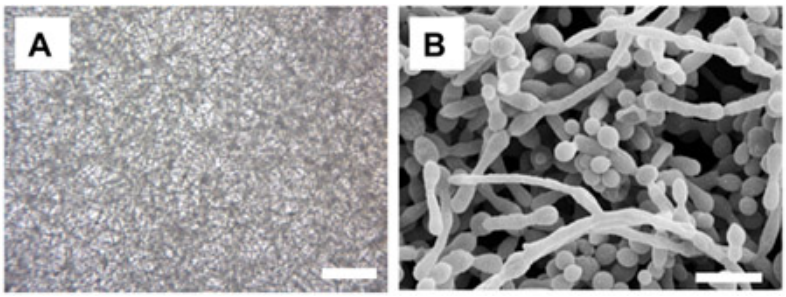

Figure 1.(A) Panel A shows a microphotograph taken using a camera attached to an inverted microscope of a *C. albicans* biofilm formed on the bottom of the well after aspiration of RPMI medium and subsequent washings with PBS. (B) A micrograph of a typical *C. albicans* biofilm visualized using scanning electron microscopy. Bars are 100 µm and 10 µm for panels A and B respectively.

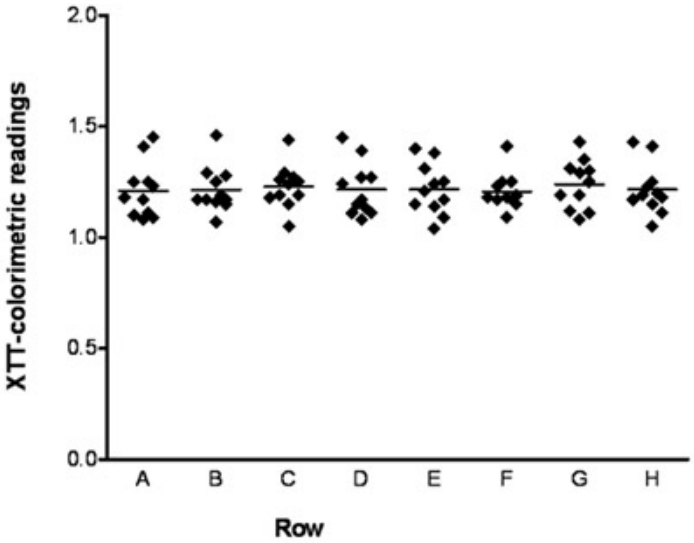

Figure 2. Formation of multiple equivalent *C. albicans* biofilms in 96-well microtiter plates. Colorimetric readings (OD_{490} values) from XTT-reduction assays of biofilms formed by a *C. albicans* wild type in wells of microtiter plates. Values are for 11 independent biofilms formed in each of 8 different rows of the same 96 well microtiter plate. 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 ($P > 0.05$).

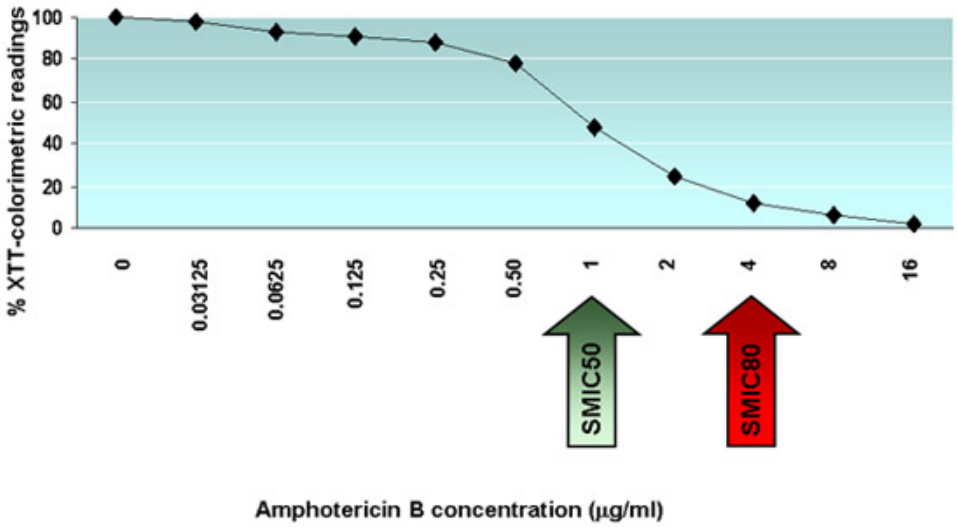

Figure 3. Typical results of antifungal susceptibility testing against *C. albicans* biofilms. Graph depicting typical results of the efficacy of different amphotericin B concentrations against biofilms of a *C. albicans* wild type strain. Values are expressed as average percent colorimetric readings for XTT-reduction assays compared to control wells. SMIC50 and SMIC80 values are indicated by arrows.

Discussion

Here we describe a simple, rapid, economical and highly reproducible 96 well microtiter plate model for the formation of *Candida* biofilms coupled with a colorimetric method that measures the metabolic activities of cells within the biofilm using XTT. This 96 well microtiter plate model for biofilm formation was originally developed for *C. albicans* but can be used for other *Candida* spp. and easily adapted for other fungal organisms. The method can be used to examine multiple parameters and factors influencing biofilm formation and to estimate the biofilm-forming ability of multiple fungal isolates and/or mutant strains. But perhaps most importantly, this method is very useful for the determination of antifungal susceptibility testing of cells within biofilms.

Disclosures

JLL-R owns equity in MicrobeHTS Technologies, Inc., which is developing antifungal agents. MicrobeHTS Technologies, Inc. provided no financial support for these studies.

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