Development of a 96-well catheter-based microdilution method to test antifungal susceptibility of Candida biofilms

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Background: Candida biofilms, which are often associated with device-related infections, including catheter-related bloodstream infections, are resistant to commonly used antifungal agents. Current microtitre (96-well) plate-based methods to determine the antifungal susceptibility of these biofilms do not involve clinically relevant substrates (e.g. catheters), and are not well suited for evaluating the surface topography and three-dimensional architecture of biofilms. We describe a simple, reproducible catheter-based microtitre plate method to form biofilms and evaluate their antifungal susceptibility.

Methods: Biofilms were formed by Candida species on 5 mm catheter discs placed in microtitre plates and quantified using metabolic conversion of a formazan dye (XTT). The morphology, surface topography and three-dimensional architecture of these biofilms were evaluated by fluorescence, confocal scanning laser and scanning electron microscopy, respectively. The optimized XTT method was used to evaluate the antifungal susceptibility of formed Candida biofilms to fluconazole, voriconazole, itraconazole and anidulafungin.

Results: Maximum XTT activity was achieved within 90 min. All tested Candida strains formed robust biofilms on catheter discs at both 24 and 48 h (P = 0.66). Biofilms exhibited typical gross morphology, surface topography and architecture, and no difference in biofilm thickness (P = 0.37). The three tested azoles were not active against the biofilms (MIC ≥ 64 mg/L), but anidulafungin possessed potent activity against them (MIC = 0.063–0.125 mg/L).

Conclusions: The developed method is simple, rapid and reproducible, and requires relatively small amounts of drug. It can be used to perform both high-resolution microscopic analysis of the topography and architecture of biofilms, and evaluation of their antifungal susceptibility.

Keywords: biofilm architecture, surface topography, resistance

Introduction

Biofilms, which are adherent microbial communities of cells embedded in a self-produced extracellular matrix, are associated with device-related infections, and significant morbidity and mortality.1–3 A characteristic feature of Candida biofilms is that they exhibit reduced susceptibility to most commonly used antifungal agents. Currently, the antifungal susceptibility of catheter-associated biofilms is evaluated using a 12-well format (employing 15 mm discs)4 or a 96-well format (in which no device substrate is present).5 Routine use of the 12-well macrodilution method in clinical laboratories and drug screening is hampered by the requirement for large amounts of the drug, the large number of 12-well plates and 15 mm catheter discs, and the long readout time. The drawback with the existing 96-well format assay for biofilms is that it does not include any substrate (e.g. catheter) and, therefore, does not mimic the clinical setting where biofilms are formed on an indwelling medical device, such as a catheter. Moreover, detailed microscopic analysis of biofilms formed directly on microtitre plates is difficult to perform.

Therefore, a standardized microdilution method that incorporates a substrate (e.g. catheter) is needed to facilitate the rapid screening of antibiofilm compounds as well as guide clinicians in choosing treatment options in cases where biofilm formation is suspected. To address this need, we developed a catheter-based microdilution method for biofilm formation using 96-well plates, 5 mm catheter discs and a shorter readout time. We used the developed method to successfully
determine the antifungal susceptibility of Candida biofilms to representative azole and echinocandin drugs.

Materials and methods

Organisms
Two Candida albicans strains (SC5314 and M61) and two Candida parapsilosis strains (PAAT1 and 514) were tested in the current study. These strains are well documented to produce abundant biofilms.\(^6\) Candida cells were maintained in glycerol stocks at \(-80^\circ\text{C}\) until needed.

Medium and growth conditions
All Candida strains were grown in yeast nitrogen base medium (YNB; BD, Sparks, MD, USA) containing 50 mM glucose (Fisher, Fairlorn, NJ, USA). Fifty millilitres of medium (in 250 mL Erlenmeyer flasks) was inoculated with Candida cells from fresh Sabouraud dextrose agar plates (BD) and incubated for 24 h at 37\(^\circ\text{C}\) in an orbital shaker. Cells were harvested and washed twice with 0.15 M PBS (pH 7.2; Ca\(^{2+}\) and Mg\(^{2+}\) free). Yeast cells were resuspended in 10 mL of PBS, counted after serial dilution using a haemocytometer, standardized to 10\(^7\) cells/mL and used to form biofilms.

Substrate material
Silicone elastomer (SE) sheets were obtained from Invotec International (Jacksonville, FL, USA). Following the manufacturer’s instructions, the catheter material sheet was cleaned by scrubbing thoroughly with a clean, soft-bristled brush in a hot water/hand soap solution, rinsed with distilled water and autoclaved. Flat circular discs, 5 mm in diameter, were obtained by cutting through the catheter sheets with a cork borer, as described previously.\(^4\)

Biofilm formation
Initially, the SE discs were pre-coated with fetal bovine serum (Mediatech, VA, USA) for 24 h at 37\(^\circ\text{C}\) on a rocker table; the pre-coated discs were then exposed to 4 mL of a Candida cell suspension (1×10\(^7\) cells/mL) (for ease of use and to ensure reproducibility, these steps were performed in 12-well plates, eight discs per well). The discs were incubated for 90 min at 37\(^\circ\text{C}\) (adhesion phase), then removed carefully with a sharp sterile needle and separately placed in each well of a 96-well flat-bottomed microtitre plate (BD) containing 200 \(\mu\text{L}\) of YNB medium. Next, in the ‘biofilm formation phase’, discs containing adherent cells were incubated for different times (24 or 48 h) at 37\(^\circ\text{C}\) on a rocker table, which allowed the formation of biofilms. All assays were carried out in at least three replicates on different days.

Quantification of biofilm
Quantification of Candida biofilms was performed using a metabolic activity assay based on the conversion of the dye 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[[(phenylamino)carbonyl]-2H-tetrazolium bromide (XTT) by mitochondrial dehydrogenase into a water-soluble formazan product that is measured spectrophotometrically.\(^4\) A working solution containing XTT and menadione (both from Sigma Chemical Co., St. Louis, MO, USA) was prepared by adding 50 \(\mu\text{L}\) of XTT from 1 mg/mL XTT stock and 4 \(\mu\text{L}\) of menadione from 1 mM menadione stock solution (diluted from 10 mM menadione stock) to 4 mL of PBS and mixing gently. YNB was carefully removed by aspiration from each well of the 96-well plate containing discs with formed biofilms and replaced with 200 \(\mu\text{L}\) of the XTT:menadione mixture. The microtitre plates were covered with aluminium foil and incubated for different times (30 min to 300 min) at 37\(^\circ\text{C}\) on a rocker. Blank wells containing 200 \(\mu\text{L}\) of the XTT:menadione mixture with discs and no biofilm were prepared, covered with aluminium foil and incubated with the biofilm plate. After removing the plates from the incubator, they were spun at 3000 rpm for 5 min using a microtitre plate centrifuge rotor. Next, the supernatant was carefully removed into a new 96-well flat-bottomed microtitre plate and the optical density (OD) read at 490 nm using a Microplate Reader (Bio-Rad, Japan).

Fluorescence microscopy
To examine biofilms using fluorescence microscopy, biofilms were grown on catheter discs and the supernatant was removed carefully by aspiration (this step is critical to avoid dislodging the formed biofilms from the catheter disc). Next, the discs containing biofilms were carefully transferred to glass microscopy slides. Two drops of calcifluor white solution (0.05% v/v; Sigma Chemical Co.) were placed on the disc, which was then kept for 1 min at room temperature.\(^7\) The stained biofilms were examined under a fluorescence microscope (Zeiss, model Axio Imager Z1m) and the images acquired quickly.

Confocal scanning laser microscopy (CSLM)
To determine the morphology and architecture of 24 h versus 48 h Candida biofilms, confocal analyses of the biofilms were performed as described previously.\(^8,9\) Briefly, after biofilms were grown for 24 or 48 h, the supernatant was removed carefully by aspiration. Next, the discs containing biofilms were transferred to a 96-well plate and incubated for 45 min at 37\(^\circ\text{C}\) in 4 mL of PBS containing the fluorescent stains FUN-1\(^\text{TM}\) (10 \(\mu\text{M}\)) and concanavalin A – Alexa Fluor-488 conjugate (ConA; 25 \(\mu\text{g}/\text{mL}\)). FUN-1\(^\text{TM}\) (excitation wavelength, 543 nm; emission, 560 nm long-pass filter) is converted into orange-red cylindrical intracellular structures by metabolically active cells, while ConA (excitation wavelength, 488 nm; emission, 505 nm long-pass filter) binds to glucose and mannose residues of cell wall polysaccharides with green fluorescence. After incubation with the dyes, the silicone elastomer discs were placed on a 35 mm glass-bottomed Petri dish (MatTek Corp., Ashland, MA, USA) and observed with a Zeiss LSM510 confocal scanning laser microscope (Carl Zeiss, Inc.).

Scanning electron microscopy
For the scanning electron microscopic examination, biofilms were formed on catheter discs and the supernatant was removed carefully by aspiration. Next, the discs containing biofilms were fixed with 2.5% (v/v) glutaraldehyde in 0.15 M PBS for 1 h at room temperature.\(^7\) They were then treated with 1% (w/v) osmium tetroxide for 1 h, washed three times with distilled water (3 mL), treated with 1% (w/v) uranyl acetate for 1 h and washed again with distilled water (3 mL). The samples were dehydrated with a series of ethanol solutions that ranged from 30% (v/v) ethanol in distilled water to dried absolute ethanol (in 10% increments). All samples were dried to critical point by using a Polaron critical-point drier, coated with gold using a Polaron coater and viewed under a Philips scanning electron microscope (Model XL30 ESEM).

Antifungal susceptibility testing of Candida biofilms
Fluconazole, voriconazole and anidulafungin were obtained from Pfizer Pharmaceuticals Group (New York, NY, USA);itraconazole was purchased from Sigma Chemical Co. Fluconazole was solubilized in sterile saline, while the remaining three drugs were solubilized in DMSO. Following biofilm formation (24 h), the spent medium was carefully aspirated out from each well. Drug dilutions in YNB broth were prepared in pre-sterilized, polystyrene, flat-bottomed 96-well microtitre plates.
biofilms formed on 5 mm discs in the microdilution method reach the mature phase after 24 h of incubation.

**Biofilms formed on 5 mm discs in microtitre plates for 24 or 48 h exhibit similar morphology, surface topography and three-dimensional architecture**

To evaluate the gross morphology of biofilms formed on 5 mm discs, we performed fluorescence microscopy using calcofluor white, an ultraviolet-excitible dye that binds chitin and β-glucan, and has previously been used to characterize the biofilm structural elements. C. albicans biofilms formed on 5 mm discs for 24 or 48 h exhibited typical fungal structural elements, with abundant hyphae enmeshed within a hazy extracellular matrix (Figure 2a and b). The hazy appearance was due to diffuse staining of the extracellular material with calcofluor white and indicated that this material is composed mainly of cell-wall-like polysaccharides. Next, we performed scanning electron microscopic analyses of the formed biofilms to determine whether their surface topography varies with growth time. We found that biofilms formed on 5 mm SE discs were composed of a dense layer of yeast and hyphal forms (Figure 2c and d). These biofilms also contained a granular extracellular matrix, which appeared as clumps due to the dehydration steps used during the processing of samples for scanning electron microscopy. Since scanning electron microscopy requires serial fixation and dehydration of samples, which can severely distort biofilm architecture, we used the non-invasive CSLM technique to examine the architecture of C. albicans biofilms using the fluorescent dyes FUN-1™ and ConA, which indicate cell viability and the presence of cell wall glucose/mannose, respectively. Our analyses revealed a highly heterogeneous architecture of both 24 and 48 h C. albicans biofilms (Figure 2e and f). Staining with ConA confirmed the presence of polysaccharide-rich extracellular material in these biofilms (diffuse green staining; Figure 2e and f). Orthogonal analyses of three-dimensional reconstructed images revealed that mature C. albicans biofilms formed on 5 mm discs had similar thickness at both 24 and 48 h (36.0 ± 3.6 μm and 41.7 ± 2.9 μm, respectively; P = 0.37). Taken together, these results demonstrate that Candida biofilms formed on catheter discs in microtitre plates for 24 or 48 h exhibit similar morphology, surface topography and three-dimensional architecture.

**Candida biofilms formed on catheter discs in microtitre plates are resistant to azoles but susceptible to anidulafungin**

Next, we used the developed catheter-based microtitre biofilm method to evaluate the antifungal susceptibilities of biofilms formed by C. albicans or C. parapsilosis. As shown in Table 1, the biofilms formed by all the tested strains exhibited resistance to fluconazole (MIC ≥256 mg/L). Both albicans and non-albicans species of Candida exhibited reduced susceptibility to itraconazole and voriconazole (MIC ≥64 mg/L for all tested strains). In contrast, biofilms formed by both C. albicans strains were susceptible to anidulafungin (MIC = 0.063 and 0.125 mg/L for strains SC5314 and M61, respectively); similar results were obtained for the two C. parapsilosis strains tested (MIC = 0.125 mg/L for both strains). These results showed that biofilms formed in our
catheter-based microtitre assay were not susceptible to azoles, but were susceptible to anidulafungin, an echinocandin.

**Discussion**

In this study, we developed a simple, rapid and reproducible method to test the antifungal susceptibility of *Candida* biofilms. Additionally, this method allows microscopic analysis of the surface topography and three-dimensional architecture of *Candida* biofilms.

For the quantification of the formed biofilms, we used a colorimetric assay that relies on the conversion of XTT tetrazolium salt into a reduced coloured formazan by mitochondrial dehydrogenases of live cells.\(^5\)\(^,\)\(^7\) Although the XTT assay has

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**Figure 2.** Morphology, surface topography and three-dimensional architecture of *Candida* biofilms grown to 24 or 48 h. Fluorescence micrographs of biofilms grown to (a) 24 h or (b) 48 h exhibited similar hyphae-rich morphology (magnification: ×10). Scanning electron micrographs of (c) 24 h and (d) 48 h biofilms showed yeast and hyphae, interspersed with granular extracellular matrix (magnification: ×3250). Confocal scanning laser microscopy of (e) 24 h and (f) 48 h biofilms exhibited a highly heterogeneous architecture of mature *C. albicans* biofilms encased in extracellular material (diffuse green staining; magnification: ×10). Metabolically active cells appear yellow due to co-staining with FUN-1\(^\text{TM}\) (red) and ConA (green) dyes. Biofilms (24 or 48 h) formed on 5 mm SE discs were composed of a dense layer of yeast and hyphal forms, embedded in extracellular polymeric material that had an amorphous granular appearance.
some limitations when comparing biofilm formation across different Candida species, it has been widely accepted as a method for quantifying biofilm formation. Our results demonstrated that the XTT assay was highly reproducible in the microtitre plate format and that peak XTT activity was reached within 90 min. Since the 12-well method requires a 300 min incubation to complete the XTT assay, adoption of the microtitre plate method developed here will allow a reduction of the time needed for the readout of results in a clinical microbiology laboratory setting. Moreover, the cost of screening investigational drugs or the evaluation of antifungal susceptibility is also reduced, since the amount of drugs used in the microtitre plate method is reduced.

Pierce et al. described a 96-well microtitre plate model in which Candida biofilms were formed directly into the wells of a 96-well plate, with no substrate present. In our study, biofilms were formed on a clinically relevant substrate (5 mm catheter discs) placed in the wells of 96-well microtitre plates. Forming biofilms on catheter discs allows the evaluation of their surface topography and three-dimensional architecture (by scanning electron microscopy and CSLM, respectively), which is not feasible with biofilms formed directly in the wells of a microtitre plate.

We used the developed method to successfully evaluate the susceptibility of biofilms formed by C. albicans and C. parapsilosis to four antifungal agents. We found that fluconazole showed poor activity against all Candida biofilms tested, while the MICs of voriconazole and itraconazole were also elevated, indicating reduced activity against biofilms. In contrast, anidulafungin showed potent activity against Candida biofilms. Our results confirmed previous reports that biofilms are resistant to azoles but susceptible to echinocandins and show that the developed method has utility in determining the antifilm activity of drugs.

In conclusion, we developed a catheter-based microtitre biofilm method that is simple, rapid, reproducible and cost-effective, and can be used for evaluating the antifungal susceptibility of biofilms. The developed method is amenable to high-throughput screening to identify compounds that inhibit biofilms. However, such a screening application will need further assay development (e.g. optimization of the substrate and determination of the assay variability ratio). Studies focused on the large-scale intra- and interlaboratory validation of the developed method are warranted.

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### Transparency declarations

None to declare.

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