Keratitis-Associated Fungi Form Biofilms with Reduced Antifungal Drug Susceptibility

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PURPOSE. To investigate the biofilm-forming capacity of *Fusarium solani*, *Cladosporium sphaerospermum*, and *Acremonium implicatum*, and the activities of antifungal agents against the three keratitis-associated fungi.

METHODS. The architecture of biofilms was analyzed using scanning electron microscopy and confocal scanning laser microscopy (CSLM). Susceptibility against six antifungal drugs was measured using the CLSI M38-A method and XTT reduction assay.

RESULTS. Time course analyses of CSLM revealed that biofilm formation occurred in an organized fashion through four distinct developmental phases: adhesion, germling formation, microcolony formation, and biofilm maturation. Scanning electron microscopy revealed that mature biofilms displayed a complex three-dimensional structure, consisting of coordinated network of hyphal structures glued by the extracellular matrix (ECM). The biofilm susceptibility testing demonstrated a time-dependent decrease in efficacy for all six antifungal agents as the complexity of fungal hyphal structures developed. Natamycin (NAT), amphotericin B (AMB), and NAT were the most effective against *F. solani, C. sphaerospermum*, and *A. implicatum* biofilm, respectively.

CONCLUSIONS. Corneal isolates of *F. solani, C. sphaerospermum*, and *A. implicatum* could produce biofilms that were resistant to antifungal agents in vitro. (Invest Ophthalmol Vis Sci. 2012; 53:7774–7778) DOI:10.1167/iovs.12-10810

Fungal keratitis is one of the most important causes of ocular morbidity and visual loss in developing nations, where it may account for nearly half of corneal ulcers. *Fusarium* and *Aspergillus* are the most common cause of fungal keratitis, followed by dematiaceous fungi.

In natural environments, fungal species are able to shift between a planktonic and a biofilm state. The biofilm is a hyphal network embedded in an extracellular matrix (ECM) and is resistant to the effects of antifungal drugs. Biofilms are considered as the most important developmental characteristics in infectious keratitis. Fungal biofilms have been found within corneas in cases of infectious crystalline keratopathy. *Fusarium* keratitis clinical isolates can form biofilms on soft contact lenses, and the biofilms induce fungal keratitis on injured corneas of mice. Moreover, clinical isolates of *Candida* and *Aspergillus* have been shown to grow and form biofilms. No study has analyzed whether *Cladosporium sphaerospermum* (a dematiaceous fungus) and *Acremonium implicatum* can grow and form biofilms, however.

In the current study, we used three fungal strains, *Fusarium solani, C. sphaerospermum*, and *A. implicatum*, isolated from patients with keratitis to compare the differences in their capabilities of biofilm formation, and to determine their antifungal susceptibility during different phases of growth and development of biofilm.

MATERIALS AND METHODS

Fungal Strains and Growth Conditions

*F. solani, C. sphaerospermum*, and *A. implicatum* were isolated from patients with fungal keratitis not associated with contact lens use at the Beijing Institute of Ophthalmology, Beijing, China, and were characterized using DNA sequence data. Fungal isolates were grown at 30°C for 7 days on potato dextrose agar. Then conidia were harvested, washed with PBS, and standardized to 1 × 10⁶ conidia/ml for biofilm formation experiments.

Biofilm Formation

To evaluate biofilm formation by *F. solani, C. sphaerospermum*, and *A. implicatum*, coverslips were washed with PBS, placed in 24-well tissue culture plates with 2 ml standardized cell suspension, and incubated for selected time periods (0, 8, 16, 24, and 48 hours) at 37°C.

Confocal Scanning Laser Microscopy

The architecture of biofilms was analyzed using confocal scanning laser microscopy (CSLM) following a previous report. Biofilms were allowed to form as described previously. After incubation at 37°C for various time periods (0, 8, 16, 24, and 48 hours), the coverslips were incubated for 30 minutes at 37°C in 2 ml of fluorescent stains FUN-1 (10 μM; Invitrogen, Carlsbad, CA) and concanavalin A Alexa Fluor 488 (ConA, 100 μg/ml; Invitrogen). FUN-1 (excitation wavelength, 559 nm; emission, 591 nm; Invitrogen) was converted to red fluorescent intravacuolar structures by metabolically active cells, whereas ConA (excitation wavelength, 488 nm; emission, 519 nm) bound to β-mannopyranosyl and α-glucopyranosyl residues and emitted green fluorescence. Yellow areas represented dual staining. Stained biofilms were observed by using the Olympus Fluoview FV1000 confocal laser scanning microscope (Olympus, Tokyo, Japan). To determine the depth of the biofilms and the overall physical ultrastructure, sections of the xy plane were taken at 2μm intervals along the z-axis. The images were captured and processed for display using FV10-ASW 2.0 software (Olympus).

Scanning Electron Microscopy

For scanning electron microscopy analysis, biofilms were allowed to form as described previously. After incubation at 37°C for 48 hours,
Biofilms Formed by Keratitis-Associated Fungi

Antifungal Susceptibility Testing of Fungal Biofilm

Susceptibility of fungal biofilms to different antifungal drugs was evaluated using 2,3-bis-[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT) reduction assay following the previous report. Briefly, conidial suspensions were incubated over selected time points (8, 16, 24, and 48 hours) at 35°C in flat-bottomed 96-well microtiter plates. After incubation, biofilms were washed twice with PBS to remove nonadherent cells. Then biofilms were exposed to each tested antifungal agent (0.125–256 mg/L) for a further 48 hours at 35°C. A series of antifungal agent-free wells and biofilm-free wells were included to serve as positive and negative controls, respectively. Following challenge, the antifungal drugs were removed and the antifungal activity was quantified by XTT reduction assay. Biofilm minimum inhibitory concentrations (MICs) were determined as 50% reduction in metabolic activity compared with the drug-free control.

Statistics

Statistical analysis was performed by using one-way ANOVA. A P value less than or equal to 0.05 was considered statistically significant.

Results

Biofilm Formation

CSLM. Morphological and structural growth patterns of *F. solani*, (Figs. 1A–E), *A. implicatum* (Figs. 1F–J), and *C. sphaerospermum* (Figs. 1K–O) were studied by CSLM. *F. solani* germ tubes were clearly seen after 8 hours of growth (Fig. 1B). Microcolonies were seen after 16 hours of growth (Fig. 1C) and biofilms were formed after 24 hours of incubation (Fig. 1D). During the maturation phase (24–48 hours), surface colonization occurred gradually (Fig. 1E). *A. implicatum* and *C. sphaerospermum* germ tubes were seen after 16 hours of growth (Figs. 1H, 1M). Microcolonies were seen after 24 hours growth (Figs. 1I, 1N). Mature biofilms were formed after 48 hours of incubation (Figs. 1J, 1O).

For CSLM examination of a 48-hour-old *F. solani*, *C. sphaerospermum*, and *A. implicatum* biofilm, a combination of the fluorescent dyes FUN-1 and ConA was used. Three-dimensional (3-D) reconstructed images were used to determine biofilm architecture and thickness (Fig. 2). Mature biofilm consisted of a highly organized structure, displaying channels between groups of hyphae. It revealed internal regions of a dense network of hyphae completely encased within a surrounding ECM.

Figure 2M shows the thickness of biofilm from *F. solani*, *C. sphaerospermum*, and *A. implicatum* biofilm, after growth for 48 hours. The thickness of *F. solani*, *A. implicatum*, and *C. sphaerospermum* biofilm reached 30 μm (±2 μm), 20 μm (±3 μm), and 25 μm (±5 μm), respectively; however, there was no significant difference in the thicknesses of biofilms among the three fungal species.

Scanning Electron Microscopy. Scanning electron microscopy was used to study the architecture of biofilms formed by *F. solani*, *A. implicatum*, and *C. sphaerospermum* after 48 hours of incubation (Fig. 3). In fungal biofilm, the surface displayed a highly coordinated network of hyphal structures that crossed each other. The ECM was observable between hyphae, where it apparently glued together the hyphal threads of the network. Channels were observed by scanning electron microscopy.

The scanning electron microscopy images in Figure 3 illustrate the different architecture of biofilms formed by *F. solani*, *C. sphaerospermum*, and *A. implicatum*. For example, *F. solani* biofilm was a mix of hyphae and a little ECM, which was patchily distributed (Fig. 3A). Moreover, *A. implicatum*...
biofilm was rich in hyphae running in every direction and an abundant ECM, which was evenly distributed (Fig. 3B), whereas biofilm formed by *C. sphaerospermum* consisted of hyphae that grew vertically to the surface of the colony and was minimally detectable ECM (Fig. 3C).

### Susceptibility of Planktonic Cells and Fungal Biofilm to Antifungal Drugs

The Table summarizes the results from susceptibility testing of planktonic cells and fungal biofilm.

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<th>BMIC50 (mg/L)</th>
<th>BMIC50 (mg/L)</th>
<th>BMIC50 (mg/L)</th>
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**Figure 2.** Confocal scanning laser microscopy images of mature fungal biofilm. *F. solani* (A–D), *A. implicatum* (E–H), and *C. sphaerospermum* (I–L) conidia were incubated for 48 hours, and then were stained with ConA and FUN1 dyes. Intensity projection over the z-axis (A, E, I), and 3-D representations of biofilm, such as top view (B, F, J), bottom view (C, G, K), and side view (D, H, L) are shown. Depths of mature fungal biofilm were quantified in triplicate (M). The error bars indicate the SEM. Original magnification: ×400. Arrowheads indicate extracellular matrix. Arrows indicate channels.

**Figure 3.** Scanning electron microscopy images of mature fungal biofilm (48 hours) grown on coverslips. Pathogens were *F. solani* (A), *A. implicatum* (B), and *C. sphaerospermum* (C). Original magnification: ×1000. Arrowheads indicate extracellular matrix. Arrows indicate channels.
AMB, ITC, and TRB were the most active agents against planktonic F. solani, C. sphaerospermum, and A. implicatum cells, respectively. As the development of biofilm, all of the antifungal agents tested showed decreased activity. Against 48-hour growth, NAT, AMB, and NAT were the most effective agents against F. solani, C. sphaerospermum, and A. implicatum biofilm, respectively.

**DISCUSSION**

In this study, we demonstrated that corneal isolates of F. solani, C. sphaerospermum, and A. implicatum can form biofilms protected from antifungal drugs through time-dependent phases in vitro. To our knowledge, this is the first study to investigate the growth characteristics of C. sphaerospermum and A. implicatum biofilms, and to examine four distinct developmental phases of the biofilms in relation to antifungal susceptibility.

Our results demonstrated that F. solani, C. sphaerospermum, and A. implicatum can produce biofilms through four basic time-dependent phases, including conidial adhesion, germ cell formation, microcolony formation, and biofilm maturation. It was consistent with those of other authors reporting on Aspergillus biofilms. It was observed that ECM was composed of galactomannan; α-1,3-glucans; monosaccharides; and polysaccharides, and melanin, and proteins. In mature biofilms, crossing hyphae were glued by ECM, which was produced between the hyphae and surrounded them, and mature biofilms displayed a three-dimensional architecture. The finding that fungal isolates varied in their ability to form biofilms and antifungal drugs exhibited varying activity against fungal biofilms was similar to a previous report. Mukherjee et al. characterized the biofilms formed by F. solani and Fusarum oxysporum, and reported species-dependent antifungal susceptibility of these two species. We presumed that different antifungal susceptibility of F. solani, C. sphaerospermum, and A. implicatum biofilm might be due to the strains. Therefore, it was necessary to adjust treatment according to fungal strains and fungal biofilm growth phases. In conclusion, F. solani, C. sphaerospermum, and A. implicatum can produce biofilms that were resistant to antifungal agents in vitro. Future studies are needed to demonstrate the potential impact of fungus biofilm on fungal keratitis.

**References**


