Original Article

Time-Lapse Tracking of Candida tropicalis Biofilm Formation and the Antifungal Efficacy of Liposomal Amphotericin B

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SUMMARY: Candida species bloodstream infection, or candidemia, remains an important health issue with high morbidity and mortality. Bloodstream infections caused by Candida species are often associated with the ability of Candida to form biofilms on medical devices, such as central venous catheters. Non-albicans Candida species have been increasing gradually in clinical settings. Another Candida species, C. tropicalis, has a propensity to form biofilms and is also an independent risk factor for high morbidity and mortality in hospitalized patients. This study was conducted to investigate the process of biofilm formation by C. tropicalis and the antifungal activity of liposomal amphotericin B (LAB) against both forming biofilms and developed biofilms using time-lapse imaging. We found that C. tropicalis has a high capacity for hyphal growth and gas generation due to its high metabolic activity. Thus, we visually observed the formation of aggressive C. tropicalis biofilms, which are fast-growing biofilms. We found that LAB acts immediately and completely inhibits forming biofilms. Furthermore, we demonstrated that LAB was effective against developed C. tropicalis biofilms by reducing the growth of hyphae and morphological changes. These results suggest that LAB may be effective for the treatment of infections caused by catheter-related C. tropicalis biofilms.

INTRODUCTION

Candida species (spp.) bloodstream infections remain a considerable cause of morbidity and mortality (1, 2). Bloodstream infections caused by Candida spp. are often related to the ability of Candida to form biofilms on medical devices, including central venous catheters (3, 4). The formation of Candida biofilms on implanted devices causes an important clinical problem because of the elevated resistance of these biofilms to antifungal agents (5, 6). In candidemia, the Candida spp. that form biofilms are associated with 1.77-fold higher mortality rates than the Candida spp. that do not form biofilms (7). If medical device removal is not feasible, systemic treatment with liposomal amphotericin B (LAB), a fungicidal agent that is effective against most Candida spp., is an alternative therapy for biofilm infection (2, 8). Mortality due to candidemia is high, and cases of both C. albicans and non-albicans Candida infections have been increasing (7-9). In particular, C. tropicalis forms biofilms, is associated with a poor prognosis (10, 11), and is a significant risk factor for death due to candidemia (12). These effects differ among regions, and C. tropicalis is the major cause of fungal infection in South East Asia, including in Japan and Taiwan (13).

The majority of studies related to biofilm formation have focused on C. albicans biofilms (14-18); however, the biofilm development process is largely unknown, with a particular lack of information concerning C. tropicalis biofilms. Furthermore, the activity of antifungals against biofilms is almost unknown.

We have previously compared the sensitivity of 3 major non-albicans spp. to antifungal agents. LAB exhibited strong activity and dose-dependent efficacy against C. tropicalis biofilms. In contrast, micafungin displayed a paradoxical growth effect and fluconazole was ineffective against the C. tropicalis biofilms. The previous study showed that Candida biofilms have distinct susceptibilities to LAB (19). To our knowledge, it is not clear how C. tropicalis biofilms are assembled or how LAB affects C. tropicalis. The aim of this study was to visually monitor the formation of C. tropicalis biofilms and the antifungal activity of LAB against both forming and developed biofilms using time-lapse photography.

MATERIALS AND METHODS

Antifungal agents: LAB was acquired from Sumitomo Dainippon Pharma (Osaka, Japan) in powder form and dissolved in sterile water.

Strains and biofilm formation: The study was performed on a C. tropicalis isolate (AMTC001414) collected from a patient admitted to Aichi Medical University Hospital in Japan. The biofilm-forming ability and antifungal susceptibility of the clinical isolates were measured, and then, the most suitable clinical strain, which had strong biofilm formation ability, was chosen for the experiments.

Small pieces of silicone tubing (Kosan, Tokyo, Japan) were placed in a specially developed chamber. C. tropicalis was inoculated on the silicone tubing, then
incubated while undergoing perfusion with medium to reproduce biofilm formation in a catheter in a clinical setting. The silicone tubing used in the study was autoclaved, soaked in serum for 24 h, and washed with phosphate-buffered saline (PBS) 3 times. The serum-coated tubing was soaked in a solution of C. tropicalis cultured in Sabouraud glucose broth overnight at 1 × 10^7/ml with shaking for 90 min to promote the adherence of the yeast. The yeast-adhered silicone tubing was washed 3 times with PBS and placed in a specially developed chamber for time-lapse imaging. The silicone tubing was incubated at 37°C with perfusion with 5% serum in Sabouraud glucose broth using SJ-1211 PERISTA pumps (ATTO, Tokyo, Japan), which were placed on both the influx and efflux sides.

Biofilms were observed both from the top and the side, and those views were recorded by time-lapse photography for 24 h at a rate of 1 frame/min. Time-lapse images were captured using a DXC-950 charge-coupled device color camera (Sony, Tokyo, Japan) equipped with a DIA photo microscope (Nikon, Tokyo, Japan). For validation, the assays were repeated at least 3 times in 3 different conditions.

**Planktonic cell and biofilm LAB susceptibility testing:** The susceptibility of planktonic cells was determined by the Clinical and Laboratory Standard Institute M27-A3 method (20), using RPMI-1640 medium with the addition of 50% fetal bovine serum (FBS). The planktonic minimum inhibitory concentration (MIC) of LAB was visually determined as the minimum antifungal concentration that caused >50% fungal damage compared to the drug-free growth control.

The susceptibility of biofilms was determined with the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) reduction assay (21, 22). FBS was added to 24-well tissue culture plates and incubated for 24 h at 37°C. After washing 3 times with PBS, the 24-well plates containing 200 µl of a suspension of 1 × 10^7 C. tropicalis cells/ml were incubated for 1.5 h at 37°C. The cells were grown overnight in Sabouraud dextrose broth as a pre-culture condition. After washing with sterile PBS to remove unattached cells, 500 µl of fresh RPMI-1640 plus 50% FBS was added and the plates were incubated for 18 h at 37°C to allow biofilm formation. The bottoms of the wells were washed with sterile PBS prior to testing with LAB from 0.25 to 128 µg/ml. After exposure to the doubling dilutions of the antifungal agent (600 µl) for 24 h at 37°C, the cells were incubated with XTT-menadione solution for 4 h at 37°C. The medium was removed, and the plates were centrifuged for 10 min at 6,000 × g to pellet the suspended cells and remove debris. The optical density of the supernatant was measured at 492 nm using a spectrophotometer (Hitachi U-1500, Tokyo, Japan). The antifungal concentration that caused a 50% reduction in the biofilms, compared with the metabolic activity of a drug-free control, was determined as the sessile MIC. Antifungal susceptibility tests of biofilms were performed 3 times.

**Activity of LAB against biofilms:** The effect of LAB on biofilms formed by C. tropicalis was microscopically observed using the following procedures. In the preparation step, uniform C. tropicalis biofilms were formed by incubation with shaking at 37°C for 18 h in Sabouraud glucose broth. LAB was added to the fluid medium-formed biofilms. We visually evaluated the effect of LAB on biofilms using time-lapse photography under the microscope with vibration stress in the presence of medium. We repeated the procedure, continuing the shaking and taking a single frame, at regular time intervals.

The medium prior to biofilm formation, the medium was treated with 32 µg/ml of LAB. To evaluate the effect of LAB on developed C. tropicalis biofilms, after shaking incubation for 6 h, the medium was treated with 64 µg/ml of LAB. The treatment doses were determined based on the value of the maximum serum concentration (Cmax approximately 30 and 70 µg/ml, respectively) when LAB was used clinically at 2.5 mg/kg as a typical dose and 5.0 mg/kg as a high dose in Japan, in order to maintain a value close to the initial maximum serum concentration of LAB in the cultures.

**RESULTS**

**Development of C. tropicalis biofilms:** We observed biofilm formation using a specially developed chamber. The yeast cells and hyphae of C. tropicalis adhered to the surface of the silicone tubing (Fig. 1A to C). C. tropicalis was present as a mixture of yeast and mycelial cells, and the clusters developed against the flow on the surface of the tubing, with colonization starting after 7 h (Fig. 1C). After 8 to 14 h, colonization had expanded on the surface of the tubing. The clusters continued to adhere and the thickness of the clusters continuously expanded (Fig. 1D to G). After 16 to 18 h, C. tropicalis biofilms were further enhanced, and generated gas due to their high metabolic activity. The tubing was covered with C. tropicalis biofilms producing a dense gas. We observed that the thickness of the layers of biofilm became the black part which obscured the light in the chamber (Fig. 1H and I). In the absence of antifungal agents, we observed a wide, dense, and time-dependent growth of hyphae.

**Susceptibility to LAB:** The planktonic cells of C. tropicalis were susceptible to LAB, with a MIC of 16 µg/ml.

The representative data from the XTT reduction assay, showing the inhibition of the growth of C. tropicalis biofilms in the presence of different concentrations of LAB, are shown in Fig. 2. LAB showed high antifungal efficacy against biofilms of C. tropicalis, with a MIC50 value of 2 µg/ml.

**Effect of LAB against forming and developed biofilms:** In the untreated controls, we observed that the hyphae of C. tropicalis extended outside colonies and yeast-like forms grew inside colonies after 3 h of incubation. After 6 h, C. tropicalis developed large biofilm clusters. After 12 h, C. tropicalis formed biofilms rapidly and widely, and the field of view was completely covered by matured biofilms (Fig. 3 A to E).

To evaluate the antifungal effect of LAB on C. tropicalis biofilm formation, LAB was added at 32 µg/ml prior to shaking incubation. LAB acted immediately and completely inhibited the development of forming biofilms; it induced changes in the internal structures and external forms of the hyphae and yeast cells of C. tropicalis (Fig. 3F and G).
After 6 h of biofilm development, LAB was added to investigate its antifungal effect against developed biofilms. Two h after the addition of LAB, it began to inhibit hyphal growth on mature biofilms. Extracellular portions of *C. tropicalis* were immediately degenerated by LAB (Fig. 3I). Although LAB did not eradicate mature biofilms, it completely inhibited further biofilm growth (Fig. 3J). At 6 h after the addition of LAB (i.e., 12 h after the start of the incubation), LAB obviously disrupted the growth of the hyphal network of the developed biofilms in comparison with that of the untreated control (Fig. 3K).

Fig. 1. Development of *C. tropicalis* biofilms observed from a side view. The medium flows from left to right (Fig. 1A to 1C). A white arrow shows that *C. tropicalis* was presented as a mixture of yeast and mycelial forms, clustered together to form biofilms against the flow on the surface of the tubing, with colonization starting after 7 h (Fig. 1C). Image enlargement (arrow) of colonization on the surface of the silicone tubing. *C. tropicalis* biofilm developed continuously (Fig. 1D to 1G). After 16 h, all of the tubing was covered with cluster of *C. tropicalis* biofilms. The silicon tubing of biofilms was thick and dense. *C. tropicalis* biofilms enhanced metabolic activity and produced gas (Fig. 1H to 1I).

Fig. 2. Activities of different concentrations of LAB against *C. tropicalis* biofilms. Graph shows the XTT activities of *C. tropicalis* normalized to control (untreated), which was taken as 100%. The result is representative of at least 3 experiments.

Fig. 3. After 6 h of biofilm development, LAB was added to investigate its antifungal effect against developed biofilms. Two h after the addition of LAB, it began to inhibit hyphal growth on mature biofilms. Extracellular portions of *C. tropicalis* were immediately degenerated by LAB (Fig. 3I). Although LAB did not eradicate mature biofilms, it completely inhibited further biofilm growth (Fig. 3J). At 6 h after the addition of LAB (i.e., 12 h after the start of the incubation), LAB obviously disrupted the growth of the hyphal network of the developed biofilms in comparison with that of the untreated control (Fig. 3K).
DISCUSSION

Previous studies reported that the MICs of some antifungal agents for biofilms were $30 \times$ to $1,000 \times$ higher than for planktonic cells. On the other hand, the MIC of LAB has less influence on biofilm formation than other antifungal classes (14).

We confirmed in this study that *C. tropicalis* has a high capacity for hyphal growth and gas generation due to its high metabolic activity. *Candida* spp. are heterogeneous with respect to biofilm structure, biofilm matrix composition, and biofilm-forming ability. It can be assumed that *C. tropicalis*, which forms aggressive, fast-growing biofilms, is defined as a high biofilm former (23).

In the XTT reduction assay, the MIC value of 4 µg/ml of LAB for biofilm cells of *C. tropicalis* was higher than the 2 µg/ml of LAB required to inhibit planktonic cells. FBS may have acted as a eutrophic biofilm cell medium, thus increasing fungal proliferation.

*Candida* biofilm formation consists of 3 steps. Firstly, *Candida* adheres to the surface of the biomaterial (early). Secondly, *Candida* propagates yeast cells, hyphal development begins, and an exopolymeric matrix that serves as a barrier to antifungal diffusion is produced (intermediate). Lastly, mature biofilm cells are released, and they pass into the bloodstream, then colonize organs and medical devices within the host (mature, dispersal) (24-26). *C. tropicalis* biofilms are morphologically different from those of other *Candida* spp. *C. tropicalis* biofilms have been characterized as having a dense extracellular matrix rich in hexosamine, whereas the *C. albicans* biofilm matrix is rich in carbohydrates and glucose. The dense, thick extracellular matrix prevents...
the penetration of drugs, thus preventing antifungal effects (27).

In this study, LAB immediately suppressed the growth of biofilms in the early stage of formation. This suggests that LAB exhibits fungicidal activity and has activity against forming biofilms; perhaps because biofilm development was insufficient, yeast cells did not develop fully and remained in an early phase of hyphal formation. These findings suggest that early treatment with LAB may effectively suppress *C. tropicalis* biofilm-related infections.

We were also able to demonstrate that LAB was effective against developed *C. tropicalis* biofilms by reducing hyphal growth and morphological changes. Although LAB completely inhibited the further growth of developed biofilms, high-dose LAB was unable to eradicate mature biofilms. Based on our results, we hypothesize that *C. tropicalis* biofilms produce extracellular matrix components that limit LAB efficacy on growing cells (28, 29). Since mature biofilms are not easy to treat, it is necessary to control biofilm development to inhibit further enhancement of biofilm formation. This study demonstrates the efficacy of LAB in reducing biofilm formation, which indicates that it is a good candidate for the prevention of *C. tropicalis* biofilm-related infections. This could meaningfully benefit hospitalized patients with medical devices that cannot be removed.

Kaneko et al. reported recently that they analyzed real-time data comprising time-lapse images to reveal the process of *Candida* biofilm development and the effects of antifungal agents on biofilms (30). However, the effects of antifungal agents on *C. tropicalis* biofilms have not been examined using time-lapse imaging. Therefore, we performed continuous observation of a shaking culture model to evaluate the activity of LAB on *C. tropicalis* biofilms. Currently, real-time observation studies of *Candida* biofilm formation still lack information about the pathogenicity and heterogeneity of the biofilms. The real-time method is complicated and time-consuming, and lacks reproducibility. This is because biofilms consist of heterogeneous cells in different developmental stages and they change dramatically according to formation conditions. Consequently, it is not easy to fully understand biofilm formation as a cluster or a mass. However, this study was visually cleared to better understand the process of *C. tropicalis* biofilm development and the effect of LAB on biofilms. Therefore, a better understanding of biofilm formation is required to improve therapies against biofilms and outcomes for patients with medical devices that cannot be removed.

In conclusion, *Candida* spp. bloodstream infections caused by catheter-related biofilms are particularly serious because biofilm cells are relatively resistant to many common antifungal agents. The novelty of this study was its demonstration of *C. tropicalis* biofilm formation and the activity of an antifungal agent using time-lapse images. The time-lapse images from our study visually display the process of biofilm development both in the early and matured biofilm stages. These results suggest that LAB has fungicidal activity against growing hyphae and yeast, which contributes to its relatively potent activity against biofilms. Thus, LAB may be effective for the treatment of infections caused by catheter-related *C. tropicalis* biofilms. Further studies are required to validate these results and to identify the differences in the biofilm formation processes of other *Candida* spp.

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**Conflict of interest** Sumitomo Dainippon Pharma is acknowledged for financial support of this study.

**REFERENCES**


