Inhibition of biofilm formation and lipase in *Candida albicans* by culture filtrate of *Staphylococcus epidermidis* in vitro

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**Abstract**

**Background:** *Candida* spp. are fourth most common cause of bloodstream infection in developed countries and emerging agents of fungemia in developing countries, with considerable attributable mortality. Candidemia is associated with the formation of complex, structured microbial communities called biofilms. Biofilm formation makes treatment difficult due to improper drug penetration and factors like high cost and adverse effects of antifungal drugs available. Hence, low-cost alternatives are urgently required to treat device-associated invasive candidiasis. **Objectives:** To study the effect of culture filtrate of *Staphylococcus epidermidis* on biofilm formation and lipase expression of *Candida albicans* in vitro. **Materials and Methods:** Yeast cells isolated from clinical samples were suspended to a turbidity of 10^6 in (a) Yeast extract-peptone-dextrose (YPD) broth and (b) culture filtrate, and 100 μl of each were dispensed in separate wells of microtiter plate. After repeated washing and reloading with respective liquid media, readings were taken spectrophotometrically. To check for lipase inhibition, yeasts were incubated overnight in YPD and filtrate and subcultured on media containing Tween-80 and CaCl_2. Positive lipase activity was denoted by haziness around colonies. **Results:** Mean reading of *C. albicans* in YPD broth was 0.579 while the same when yeasts were suspended in *S. epidermidis* culture filtrate was 0.281 (P < 0.05 by Z-test of significance). Lipase of *C. albicans* was inhibited by culture filtrate. Filtrate was found to be nontoxic to human cell line. **Conclusions:** Culture filtrate of *S. epidermidis* can hence pave the way for development of new strategies to inhibit biofilm formation in device-associated candidemia.

**Key words:** Biofilms, *Candida* spp., *Staphylococcus epidermidis*

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**Introduction**

*Candida* spp. is a common cause of device associated bloodstream infection in developed and developing countries.[1] This disease has a tremendous attributable mortality in the order of 30–40% according to available scientific literature.[2] Invasive candidiasis is generally associated with the formation of complex microbial communities, also known as biofilms over indwelling intravascular devices.[3] Biofilms are sessile communities consisting of microcolonies of yeast cells and an exopolymeric noncellular polysaccharide matrix secreted by the yeasts.[5] *Candida albicans* is the most common species of in the genus *Candida* implicated in invasive candidiasis, in at least 5070% cases, although other species are also responsible.[3] Invasive candidiasis makes therapy very difficult, owing to factors like defective penetration of antifungal drugs through biofilms by forming a reaction-diffusion barrier and high cost of drugs available, like the echinocandins.[6,7] Moreover, antifungal drugs like amphotericin B have major adverse effects like nephrotoxicity and other ill-effects on health.[8] Intravascular catheters and other devices colonized with *C. albicans* can be removed, but this is not always feasible and antifungal treatment should be an adjunct to it.[9] Hence, low-cost safer alternatives are the need of the hour for treatment of device-associated invasive candidiasis. Lipase enzyme expressed by *C. albicans* is one of the major virulence
factors of the pathogen and its inhibition can be a strategy to abrogate infection by this pathogen.[10]

The present study was designed to evaluate the effect of filtrate of culture supernatant of *Staphylococcus epidermidis* on the biofilm formation and lipase expression of *C. albicans* *in vitro*.

**Materials and Methods**

**Type, time, design and place of study**

This was a laboratory-based observational study, carried out in the Department of Microbiology, King George's Medical University (KGMU), Lucknow, Uttar Pradesh, India. The study was conducted from July 2011 to June 2013.

**Isolation and identification of microorganisms**

Routine microbiological culture medium (5% sheep blood agar plate) was used to grow *S. epidermidis* isolates from different samples such as pus, blood, urine, and others. To isolate *C. albicans* from various clinical samples such as blood, pus, and urine, Saboraud’s dextrose agar slant with Emmon’s modification (pH 7.0) was used. Ten isolates each of *C. albicans* and *S. epidermidis* were randomly selected for the study. *S. epidermidis* isolates were identified by observing Gram-positive cocci microscopically after performing Gram-stain from the colonies on solid plates, positive catalase, and negative tube and slide coagulase tests and also a negative mannitol fermentation reaction.[11,12] *C. albicans* isolates were identified by positive germ tube test and production of a single terminal chlamydospores on Corn Meal agar plate (Dalmau slit inoculation technique) after aerobic incubation at 25°C for 48 h.[13]

**Test for biofilm formation in Candida albicans**

The microtiter plate model, as proposed by Ramage et al., was employed for biofilm formation and its inhibition *in vitro*. At first, yeast isolates were grown in YPD Broth (1% yeast extract, 2% peptone, 2% dextrose, w/v) overnight at 37°C. *S. epidermidis* isolates were suspended in YPD Broth (1 loopful of the colony in 2 ml broth) and centrifuged at 3000 rpm for 5 minutes. After that, the supernatant was filtered by passing it through the membrane filter of pore size 0.22 μm (Micro‑Por Minigen Syringe Filter; Genetix Biotech Asia, New Delhi). Then yeast cell turbidity was adjusted to 10^4 cells/ml in (a) YPD broth, (b) *S. epidermidis* culture filtrate. Then 100 μl of each set of suspension was dispensed in separate wells of a flat-bottomed 96-well polystyrene microtiter plate (Nunclon A/S, Kampstrupvej, Denmark). Sterile physiological (0.85%) saline was added in a well as a negative control. After incubating for 90 min at 37°C, the wells were washed thrice with phosphate-buffered saline (PBS, pH 7.2) to remove non-adherent yeast cells and wells were reloaded with respective sterile liquid substrates. Washing and reloading was repeated at intervals of 24 h and 48 h. After 48 h, wells were washed thrice with PBS and stained with 100 μl of 1% safranine (weight/volume) in 95% ethanol for 1 min. After washing off excess stain with PBS, the wells were observed under inverted microscope under ×200 magnification.[14] Subsequently their readings (optical densities) were also measured spectrophotometrically at a wavelength of 450 nm ultra violet light (iMark MicroPlate reader; Bio‑Rad, USA).

The first round of tests was carried out with *C. albicans* ATCC 90028 strain and then with randomly selected clinical isolates. All tests were carried out 3 times.

**Test for lipase inhibition**

The test for inhibition of lipase was carried out by subculturing yeasts incubated overnight in (a) YPD and (b) culture filtrate on Muhsin’s solid medium containing Tween-80 and CaCl_2.[15]

A positive lipase activity was defined by a zone of haziness around yeast colonies on the medium.

**Toxicity assay**

The toxic effects of the filtrate were studied by inoculating 100 μl of the filtrate on Hep-2 (human laryngeal epithelioma) cell line monolayer in small polystyrene vials, incubating it for 1 h at 37°C, washing thrice with PBS, reloading the vials with 2 ml eagle’s minimum essential medium, reincubating at 37°C, and periodic observation of the monolayer at 6 hourly intervals under an inverted microscope (×40 magnification). An uninoculated monolayer was kept as control. Experiments were repeated 3 times.

**Results**

As observed by both methods (microscopically and spectrophotometrically), biofilm formation in *C. albicans* was significantly reduced by crude culture filtrate of *S. epidermidis*, *in vitro*. The difference in mean values (optical density [OD] readings) of yeasts in YPD and the culture filtrate were calculated by Z-test of significance.[14] The differences were found to be highly statistically significant. Mean OD of *Candida tropicalis* in YPD and culture filtrate were 0.579 and 0.281, respectively (*P* < 0.05). The results were found to be reproducible when performed in triplicate. The values have been shown in Table 1.

Lipase activity was found to be inhibited by culture filtrate of *S. epidermidis*. There was no zone of haziness around colonies subcultured from the culture filtrate in repeated experiments [Figures 1 and 2].

There was no observable change in morphology or cytopathic effect on of Hep-2 cells inoculated with the culture filtrate after periodic observation for 2 days compared to control vial. Thus, the crude filtrate was found to be nontoxic to host cells [Figures 3 and 4].
Discussion

Invasive candidiasis is now regarded as the fourth most common cause of hospital-acquired bloodstream infection in the United States.[17] Very high incidence of nosocomial candidemia has also been reported from developing countries like Brazil.[18] In a study from North India, the incidence of candidemia was found to be about 45% among patients admitted in intensive care units.[19] Thus, the burden of this disease is considerable in both developed and developing countries. Candidemia is primarily caused by \textit{C. albicans}, according to scientific literature available worldwide.[20] However, species other than \textit{C. albicans} are also emerging as agents causing candidemia, and a report from North India indicated that \textit{C. tropicalis} is the most common species associated with the condition.[21] This disease entity is associated with the formation of complex microbial communities called biofilms over indwelling devices like intravascular catheters.[22] Formation of biofilms renders treatment difficult due to improper penetration of antifungal drugs through biofilms and slow growth of biofilm-associated cells.[23,24] Antifungal drugs available also have their own toxic effects which limit their routine use to treat this condition. For example, conventional amphotericin B is notorious for causing nephrotoxicity and hypokalemia and newer deoxycholate formulation is more expensive than the conventional one.[25] The echinocandins are effective against biofilms, but are prohibitively costly.[7] Hence, focus of researchers has shifted toward discovery of newer, low-cost, and safer alternatives in order to treat biofilm-associated candidemia. In this regard, it is worthy to mention that organochlorine

Table 1: Optical density reading of \textit{C. albicans} in YPD and culture filtrate

| \textit{C. albicans} | In YPD | In culture filtrate |
|----------------------|--------|---------------------|
| Experiment 1         | 1.176  | 0.831               |
| Experiment 2         | 0.016  | 0.005               |
| Experiment 3         | 0.546  | 0.008               |

\(P<0.05\) by Z-test of significance. \textit{C. albicans: Candida albicans; YPD: Yeast extract-peptone-dextrose}
derivatives (Aspirochlorine) derived from *Aspergillus flavus* have been shown to inhibit *C. albicans* growth in vitro. Similar inhibition of candidal biofilm formation has been shown by pyocyanin and lipopolysaccharide of *Pseudomonas aeruginosa.*

In a mixed environment, the slime produced by *S. epidermidis* has been documented to facilitate adhesion of *C. albicans* to indwelling devices. Conversely, *C. albicans* also shield the bacterium from the action of vancomycin. However, there is no study evaluating the effect of secreted substances by *S. epidermidis* broth culture on candidal biofilm formation and lipase expression. Similar inhibition has been shown when yeasts were grown in culture filtrate of *A. flavus.* This filtrate was found to be nontoxic and hence can be precoated over the surface of indwelling devices to impair biofilm formation by *C. albicans.* Further studies are required in this context to characterize the inhibitory substances in the crude filtrate and further check for host toxicity on animal systems.

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