Original Article

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

Sayan Bhattacharyya, Prashant Gupta[1], Gopa Banerjee[1], Amita Jain[1], Mastan Singh[1]

Departments of Microbiology, AIIMS, Phulwari Sharif, Patna, Bihar, and [1]KGMU, Lucknow, Uttar Pradesh, India

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

Submission: 27-07-2013 **Accepted:** 28-03-2014

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.[4] *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.[5] 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

Address for correspondence: Dr. Sayan Bhattacharyya, Department of Microbiology, AIIMS, Phulwari Sharif, Patna - 801 505, Bihar, India.
E-mail: sayan.bhattacharyya@yahoo.com

Access this article online	
Quick Response Code:	Website: www.ijabmr.org
	DOI: 10.4103/2229-516X.140721

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*.[14] 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 10^6 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.[16] 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 *C. albicans*, according to scientific literature available worldwide.[20] However, species other than *C. albicans* are also emerging as agents causing candidemia, and a report from North India indicated that *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 *C. albicans* in YPD and culture filtrate

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. *C. albicans: Candida albicans*; YPD: Yeast extract-peptone-dextrose

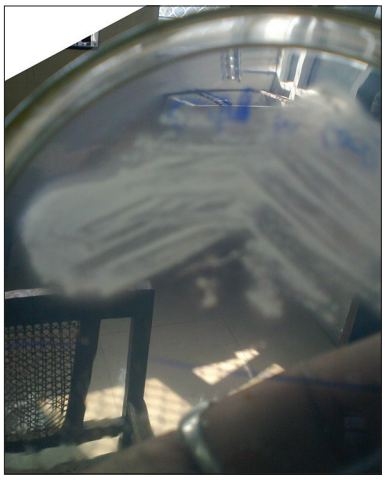

Figure 1: Haziness around Candida albicans colonies subcultured from the culture filtrate of Staphylococcus epidermidis

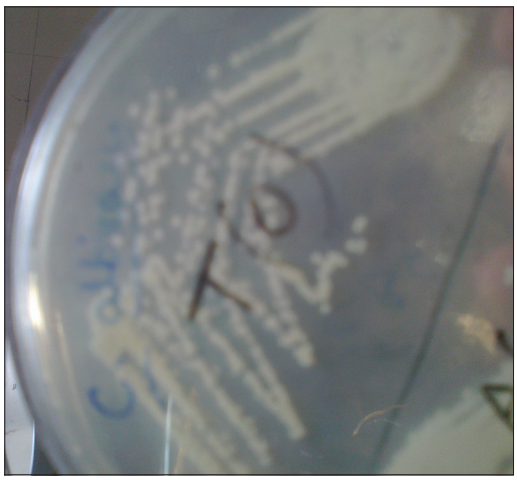

Figure 2: No haziness around colonies subcultured from yeast extract-peptone-dextrose (without Staphylococcus epidermidis filtrate)

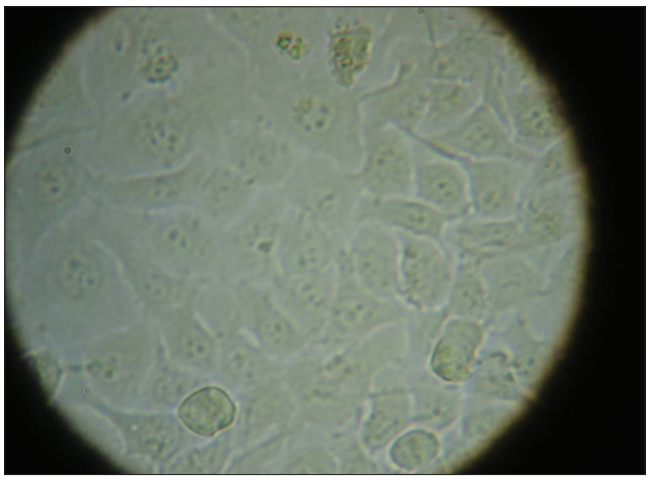

Figure 3: Hep-2 cell line incubated with Staphylococcus epidermidis culture filtrate

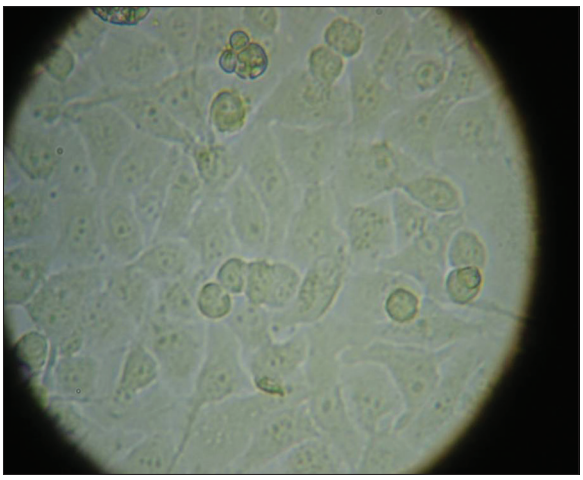

Figure 4: Similar picture (here also no cytopathic effect) when Hep-2 cell line is incubated with negative control (saline)

derivatives (Aspirochlorine) derived from *Aspergillus flavus* have been shown to inhibit *C. albicans* growth *in vitro*.[26] Similar inhibition of candidal biofilm has been shown by pyocyanin and lipopolysaccharideof *Pseudomonas aeruginosa*.[22,27]

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.[28] 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*.[29] 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.

Acknowledgment

The authors would like to acknowledge the overall help of Dr. Deepak Kumar, Junior Resident in identification of microorganisms and Mr. Mayank Agnihotri, Junior Technician, Department of Microbiology, KGMU, Lucknow for helping in reading optical density.

References

1. Reagan DR, Pfaller MA, Hollis RJ, Wenzel RP. Evidence of nosocomial spread of *Candida albicans* causing bloodstream infection in a neonatal intensive care unit. Diagn Microbiol Infect Dis 1995;21:191-4.
2. Gudlaugsson O, Gillespie S, Lee K, Vande Berg J, Hu J, Messer S, *et al*. Attributable mortality of nosocomial candidemia, revisited. Clin Infect Dis 2003;37:1172-7.
3. Taff HT, Nett JE, Zarnowski R, Ross KM, Sanchez H, Cain MT, *et al*. A *Candida* biofilm-induced pathway for matrix glucan delivery: Implications for drug resistance. PLoS Pathog 2012;8:e1002848.
4. Chandra J, Kuhn DM, Mukherjee PK, Hoyer LL, McCormick T, Ghannoum MA. Biofilm formation by the fungal pathogen *Candida albicans*: Development, architecture, and drug resistance. J Bacteriol 2001;183:5385-94.
5. Pfaller MA, Pappas PG, Wingard JR. Invasive fungal pathogens: Current epidemiological trends. Clin Infect Dis 2006;43 Suppl: 3-14.
6. Al-Fattani MA, Douglas LJ. Penetration of *Candida* biofilms by antifungal agents. Antimicrob Agents Chemother 2004;48:3291-7.
7. Morris MI, Villmann M. Echinocandins in the management of invasive fungal infections, part 1. Am J Health Syst Pharm 2006;63:1693-703.
8. Laniado-Laborín R, Cabrales-Vargas MN. Amphotericin B: Side effects and toxicity. Rev Iberoam Micol 2009;26:223-7.
9. Raad I, Hanna H, Maki D. Intravascular catheter-related infections: Advances in diagnosis, prevention, and management. Lancet Infect Dis 2007;7:645-57.
10. Yang YL. Virulence factors of *Candida* species. J Microbiol Immunol Infect 2003;36:223-8.
11. Jones D, Deibel RH, Niven CF Jr. Identity of *Staphylococcus epidermidis*. J Bacteriol 1963;85:62-7.
12. Chamberlain N. Coagulase Test for *Staphylococcus* Species. ASM Microbe Library, 2009 Aug 25. Available from: http://www.microbelibrary.org/library/laboratary-test/3207coagulase-test-for-staphylococcus-species [Accessed on 2014 July 22].
13. Ananthanarayan R, Paniker CKJ. Systemic and opportunistic mycoses. In: Anathanarayan and Paniker's Textbook of Microbiology. 9th ed. Hyderabad, India: Universities Press; 2013. p. 612.
14. Ramage G, Vande Walle K, Wickes BL, López-Ribot JL. Standardized method for *in vitro* antifungal susceptibility testing of *Candida albicans* biofilms. Antimicrob Agents Chemother 2001;45:2475-9.
15. Muhsin TM, Aubaid AH, al-Duboon AH. Extracellular enzyme activities of dermatophytes and yeast isolates on solid media. Mycoses 1997;40:465-9.
16. Mahajan BK. Sampling variability and significance. In: Methods in Biostatistics for Medical Students and Research Workers. 7th ed. New Delhi: Jaypee Brothers Medical Publishers Pvt. Ltd.; 2010. p. 114.
17. Warnock DW. Trends in the epidemiology of invasive fungal infections. Nihon Ishinkin Gakkai Zasshi 2007;48:1-12.
18. Colombo AL, Nucci M, Park BJ, Nouér SA, Arthington-Skaggs B, da Matta DA, *et al*. Epidemiology of candidemia in Brazil: A nationwide sentinel surveillance of candidemia in eleven medical centers. J Clin Microbiol 2006;44:2816-23.
19. Kotwal A, Biswas D, Sharma JP, Gupta A, Jindal P. An observational study on the epidemiological and mycological profile of Candidemia in ICU patients. Med Sci Monit 2011;17:CR663-8.
20. Asmundsdóttir LR, Erlendsdóttir H, Gottfredsson M. Increasing incidence of candidemia: Results from a 20-year nationwide study in Iceland. J Clin Microbiol 2002;40:3489-92.
21. Xess I, Jain N, Hasan F, Mandal P, Banerjee U. Epidemiology of candidemia in a tertiary care centre of north India: 5-year study. Infection 2007;35:256-9.
22. Bhattacharyya S, Gupta P, Banerjee G, Jain A, Singh M. Inhibition of *Candida* biofilms by pyocyanin. Int J Curr Res Rev 2013;5:31-6.
23. Gilbert P, Maira-Litran T, McBain AJ, Rickard AH, Whyte FW. The physiology and collective recalcitrance of microbial biofilm communities. Adv Microb Physiol 2002;46:202-56.
24. Stewart PS. Multicellular nature of biofilm protection from antimicrobial agents. In: McBain A, Allison D, Braiding M, Rickard A, Verran J, Walker J, editors. Biofilm Communities: Order From Chaos? 1st ed. Cardiff, United Kingdom: BioLine; 2003. p. 181-9.
25. Nucci M, Loureiro M, Silveira F, Casali AR, Bouzas LF, Velasco E, *et al*. Comparison of the toxicity of amphotericin B in 5% dextrose with that of amphotericin B in fat emulsion in a randomized trial with cancer patients. Antimicrob Agents Chemother 1999;43:1445-8.
26. Klausmeyer P, McCloud TG, Tucker KD, Cardellina JH 2nd, Shoemaker RH. Aspirochlorine class compounds from *Aspergillus flavus* inhibit azole-resistant *Candida albicans*. J Nat Prod 2005;68:1300-2.
27. Bandara HM, K Cheung BP, Watt RM, Jin LJ, Samaranayake LP. *Pseudomonas aeruginosa* lipopolysaccharide inhibits *Candida albicans* hyphae formation and alters gene expression during biofilm development. Mol Oral Microbiol 2013;28:54-69.
28. Adam B, Baillie GS, Douglas LJ. Mixed species biofilms of *Candida albicans* and *Staphylococcus epidermidis*. J Med Microbiol 2002;51:344-9.
29. Bhattacharyya S, Gupta P, Banerjee G, Jain A, Singh M. *In-vitro* Inhibition of Biofilm Formation in *Candida albicans* and *Candida tropicalis* by heat stable compounds in culture filtrate of *Aspergillus flavus*. J Clin Diagn Res 2013;7:2167-9.

How to cite this article: Bhattacharyya S, Gupta P, Banerjee G, Jain A, Singh M. Inhibition of biofilm formation and lipase in *Candida albicans* by culture filtrate of *Staphylococcus epidermidis in vitro*. Int J App Basic Med Res 2014;4:27-30.

Source of Support: Nil. **Conflict of Interest:** None declared.