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INHIBITION OF *CANDIDA* BIOFILMS BY PYOCYANIN: AN IN-VITRO STUDY

Bhattacharyya S., Gupta P, Banerjee G., Jain A., Singh M.

Department of Microbiology, King George's Medical University (KGMU), Lucknow, Uttar Pradesh, India

E-mail of Corresponding Author: sayantheboss@yahoo.co.in

ABSTRACT

Objectives: Invasive candidiasis has an attributable mortality of 10-49%. It is associated with biofilm formation over indwelling devices. Biofilm-associated upregulated drug efflux makes treatment expensive and ineffective. Hence low-cost alternatives inhibiting Candidal biofilm formation are needed. *Pseudomonas aeruginosa* inhibits growth of *Candida albicans* in vitro. This study aimed to detect whether secreted products of *Pseudomonas aeruginosa*, especially Pyocyanin, affect biofilm production by *Candida albicans* and *C.tropicalis*. **Methods:** *P. aeruginosa* strains were incubated overnight at 37°C in Luria broth and centrifuged. Supernatant was filtered by syringe filter (pore size 0.22µm). Yeast isolates were grown overnight in YPD broth (Yeast Extract-Peptone-Dextrose). Turbidity was adjusted to 10^6 cells/ml in YPD and culture filtrate. Then 100 µl of both suspensions were dispensed in wells of flat-bottomed 96-well microtitre plate with normal saline as negative control. Wells were washed after incubation of 90 minutes at 37°C with Phosphate buffered saline (PBS) and reloaded with 100 µl of respective liquid media. This was repeated after intervals of 24 and 48 hours. Wells were stained with 1% safranine in 95% ethanol, washed with PBS and observed under inverted microscope. Optical density was measured spectrophotometrically. Methods were repeated with filtrate, preheated at 100°C for 20 minutes and Pyocyanin extracted from *P. aeruginosa* broth culture with the help of Chloroform and acidified water. **Results**: Biofilm formation of *Candida albicans* and *C. tropicalis* was significantly reduced by culture filtrate, both plain and heated, and Pyocyanin. Pyocyanin was found non-toxic to host cells. **Conclusions:** Pyocyanin can be utilised in vivo to inhibit device-associated biofilm formation by these pathogens.

Key words: Biofilm, *Candida* spp., *Pseudomonas aeruginosa*, Pyocyanin.

INTRODUCTION

Invasive candidiasis is a major disease concern in developing countries. *Candida albicans* is the most commonly isolated species [Colombo *et al.*, 2003]. However, in regions like South America, *Candida parapsilosis* and *C. tropicalis* are the leading agents of candidemia [Colombo *et al.*, 2003]. This disease entity has an attributable mortality in the order of 40-49% without treatment, which varies from region to region [Ahmad *et al*, 2012]. It is associated with formation of complex microbial communities known as biofilms over indwelling devices like central venous catheters[Sun *et al.*, 2012].Treatment of invasive candidiasis is difficult owing to biofilm-mediated increased drug efflux[Jabra-Rizk *et al.*, 2004]. Among the antifungal agents available, only Echinocandins and Amphotericin B lipid formulations have shown consistent activity against Candidal biofilms [Kuhn et al, 2002]. However, Amphotericin B can be severely nephrotoxic to the host and Echinocandins are prohibitively costly to be used routinely [Deray, 2002 and

Morris and Vilman, 2006]. Hence, there is urgent need of less costly natural products and other alternatives against invasive candidiasis .Some workers have studied the effect of secreted products of *Pseudomonas aeruginosa* on inhibition of biofilm formation by *Candida albicans* in vitro [Holcombe *et al*, 2010]. Keeping these things in mind, our study was aimed at detecting the effect of culture filtrate of *P. aeruginosa*, both plain and heated, and Pyocyanin, in particular on biofilm formation by *Candida albicans* and *Candida tropicalis* in vitro.

MATERIALS AND METHODS

This was a laboratory-based observational study, carried out in the Department of Microbiology, KGMU, Lucknow, Uttar Pradesh, India. The study was conducted from July 2011 to June 2012.

Isolation of microorganisms:-

Routine microbiological culture media like 5% sheep blood agar and MacConkey agar plates were used to culture Pseudomonas *aeruginosa* from different samples like pus, sputum, urine and others. To isolate *Candida* spp. from various clinical samples like blood, pus and urine, Saboraud's Dextrose Agar (SDA) with Emmon's modification (pH 7.0) was used. Ten (10) each of *C. albicans* and *C. tropicalis* and 10 isolates of *P. aeruginosa* were randomly selected for the study. *P. aeruginosa* isolates were identified by Non Lactose-fermenting colonies on MacConkey Agar, positive Oxidase reaction and Citrate utilisation tests and blue green diffusible pigment(pyocyanin) production along with fruity (corn-taco) odour of the colonies on solid media[Ferguson et al., 2007, and Gaby and Hadley, 1957]. *Candida albicans* isolates were identified by positive Germ tube test, growth above 42°C and terminal chlamydospore production on Corn meal agar with Tween 80(Dalmau technique) at 25°C after 48 hours of incubation[Raju and Rajappa, 2011]. *Candida tropicalis* isolates were identified by wavy pseudohyphae along with budding yeasts on corn meal agar with Tween 80 at 25°C after 48 hours, positive fermentation of Glucose, maltose and sucrose (all in 2% concentration, w/v)but not lactose and negative germ tube test [Fungal descriptions].

Test for Biofilm formation in *Candida* spp.:-

The microtitre plate model, as proposed by Ramage *et al*, was employed for biofilm formation and its inhibition in vitro [Ramage *et al.*, 2001]. At first, yeast isolates were grown in YPD Broth (1% Yeast Extract, 2% Peptone, 2% Dextrose, w/v) overnight at 37°C. *Pseudomonas aeruginosa* isolates were incubated overnight in LB or Luria Broth (1 colony suspended in 2 ml LB) and then centrifuged at 3000 rpm for 5 minutes. After that, the culture supernatant was filtered by passing through a membrane filter (syringe filter) of pore size 0.22 µm (Micro-Por Minigen Syringe Filter, Genetix Biotech Asia, New Delhi).This filtrate was divided into 2 parts. One part was left unheated and the other was heated to 100°C for 20 minutes in a water bath and subsequently cooled. Then yeast cell turbidity was adjusted to 10^6 cells/ml in- a) YPD, b) *P. aeruginosa* unheated filtrate, and c) *P. aeruginosa* heated filtrate. About 100 µl of each set of suspension was dispensed in separate wells of a flat-bottomed 96-well microtitre plate (Nunclon A/S, Kampstrupvej, Denmark). Sterile normal saline was added in a well as negative control. After incubating for 90 minutes at 37°C, the wells were washed thrice with Phosphate-buffered saline (PBS, pH 7.2) to remove non-adherent cells and wells were reloaded with respective sterile liquid substrates. Washing and reloading was repeated at intervals of 24 hours and 48 hours. After 48 hours, wells were washed thrice with PBS and stained with 100µl of 1% safranine (w/v) in 95% ethanol for 1 minute. After washing off excess stain with PBS, the wells were observed under inverted microscope under 200X magnification [Ramage *et al.*, 2001]. Subsequently their readings (optical densities) were also measured spectrophotometrically at a wavelength of 450 nm UV light (iMark MicroPlate reader, Bio-Rad, USA). The first round of tests were carried out with *Candida albicans* ATCC 90028 and *C. tropicalis* ATCC 750 strains and then with randomly selected clinical isolates. All tests were carried out three times (in triplicate). In a second set of experiment, Pyocyanin was extracted from *Pseudomonas aeruginosa* isolates by the method described by Vinckx and co-workers [Vinckx *et al.*, 2001]. Briefly, 1 loopful Pseudomonas aeruginosa colony was grown overnight at 37°C in 5 ml Peptone water containing 1% Glucose(w/v), and then centrifuged at 3000 rpm for 5 minutes. To the cell free supernatant, equal volume of chloroform (Ranbaxy SRL, New Delhi, India) was added and it was again incubated at 37°C overnight after thorough shaking. After that the chloroform phase was purified from the bottom and to it, equal volume of acidified distilled water (pH: 4) was added. After another incubation overnight at 37°C , the watery phase was separated out in a sterile test tube and its pH brought to 7.0 (neutral pH) by adding 1 M NaOH to it. The resultant solution was purified extracted pyocyanin and its fluorescence was checked in an Ultraviolet hood. Yeast isolates were suspended in- **a)** YPD, and **b)** pyocyanin and turbidity of both was adjusted to 10^6 cells/ml. Then 100 µl of both suspensions were dispensed in wells of Microtitre plate and incubated similarly at 37°C. Wells were washed with PBS and reloaded with respective liquids. After final washing and staining, wells were observed microscopically and readings were noted spectrophotometrically at 450 nm wavelength. The toxic effects of pyocyanin, if any, were observed by inoculating shell vials coated with Hep-2 (Human laryngeal epithelioma) cell line monolayer, incubating at 37°C and observing vials under inverted microscope every 6 hours.

RESULTS

As observed by both the methods (microscopically and spectrophotometrically), biofilm formation in *Candida albicans* and *C. tropicalis* was significantly reduced by crude culture filtrate of *P. aeruginosa*, both heated and unheated, in vitro. The difference in mean values (O.D. readings) of yeasts in YPD and the culture filtrate were calculated by Z-test of significance [Mahajan, 2010]. The differences were found to be highly statistically significant. Mean O.D. of *Candida tropicalis* in YPD, unheated and heated filtrates were 3.43, 0.14 and 0.165 respectively ($p<0.05$). The corresponding readings for *Candida albicans* were 3.6, 0.12 and 0.188 respectively ($p<0.05$). The results were reproducible when the experiments were done in triplicate. The results have been shown in Table 1.

Pyocyanin also showed significant inhibition of biofilms of *Candida albicans* and *Candida tropicalis.* The mean O.D. of C. albicans in YPD was 1.515 while that in Pyocyanin was 0.081. The corresponding figures for Candida tropicalis were 0.939 and 0.080 respectively. The results have been shown in Table 2.

DISCUSSION

Invasive candidiasis is now considered the fourth most common cause of bloodstream infection(BSI) worldwide, representing 11% of all cases of BSI [Festekjian *et al.*, 2010]. Attributable mortality in this condition ranges from 10-54%, other than factors like significant morbidity and increased hospital stay [Festekjian et al., 2010 and Eggimann *et al*., 2011]. Among all the species belonging to the genus *Candida*, *C. albicans* is the most common agent causing invasive disease in Europe, but in North and South America as well as in India, species other than *C. albicans* have become commoner

[Eggimann *et al*., 2011, and Kothari and Sagar, 2009]. Invasive candidiasis is associated with formation of complex, structured microbial communities also known as biofilms, on indwelling devices such as intravascular catheters [Uppuluri *et al*., 2011]. In fact, studies have shown that *Candida* spp. is the commonest fungal species associated with biofilm formation [Ramage *et al*., 2005]. Treatment is difficult in these settings, owing to factors like ability of cells within biofilms to withstand host immune defences, defective drug penetration through biofilms and upregulated drug efflux pumps [Jabra-Rizk et al., 2004, Ramage *et al*., 2005, Matinez and Fries, 2010]. Therapy can be initiated with Amphotericin B and Echinocandins, although this is often problematic since the former has been documented to be nephrotoxic in about 50% cases and the latter group is too expensive to be used in regular practice [Deray, 2005, Morris and Villman, 2006]. Hence there is imminent need of developing low-cost compounds and natural products that inhibit biofilm production and resultant pathology in invasive candidiasis. A few naturally derived compounds, like secreted products of *Aspergillus flavus* (Aspirochlorine or organochlorine derivatives), have been found to inhibit growth of *Candida albicans* [Klausmeyer *et al.*, 2005]. Recent research has also shown that secreted products from *Pseudomonas aeruginosa* inhibit biofilm formation in *Candida albicans* [Holcombe *et al*., 2008]. These compounds, being non-toxic to the host cells as found in our sudy, can be precoated on the surface of indwelling devices, to disrupt biofilm formation in *Candida* spp. in vivo too. Furthermore, in the face of emerging candidemia due to *Candida* spp. other than *C. albicans*, the efficacy of *P. aeruginosa* culture filtrate and especially Pyocyanin in inhibiting biofilm of *C. tropicalis* is also important. Other workers have reported that *Pseudomonas aeruginosa* growth itself can inhibit growth and biofilm formation in *Candida albicans* and *C. tropicalis* when cocultured in vitro [Bandara *et al*., 2010]. Since *C. tropicalis* is becoming increasingly resistant to fluconazole and voriconazole in the Indian scenario at least, the effectiveness of these secreted factors in the filtrate implies that it can be used in biofilm-associated *C. tropicalis* infection [Kothavade *et al*., 2010, Eggimann et al., *2011*]. Such inhibition of Candidal biofilm formation has also been reported in other bacterial products. Some authors have shown such inhibition by bacterial Lipopolysachharide (LPS) of *Serratia* spp. and *Klebsiella* spp., although LPS of some Gram negative bacilli can also enhance Candidal biofilm formation in some cases [Bandara et al., 2010]. In case of our study, this inhibitory effect of culture filtrate of *P. aeruginosa* was found to be unaltered by high temperature, and hence can be used in febrile states also. This inhibitory effect was presumably due to Pyocyanin, a phenazine compound secreted by *Pseudomonas aeruginosa*. In a mixed environment, Farnesol, a quorum-sensing molecule produced by *Candida albicans* has been shown to downregulate Pyocyanin production by *P. aeruginosa* by significantly downregulating the transcription of the *pqsA* gene. This is possibly a mechanism by which the yeast pathogen protects itself from the toxic effects of Pyocyanin, an iron scavenger and a putative virulence factor of *P. aeruginosa* [Peters et al., 2012]. So far, the toxic effects of Pyocyanin on Candidal biofilm have not been studied and this study, to the best of our knowledge, is the first one in this regard. The factor in crude filtrate of *Pseudomonas aeruginosa* inhibiting Candidal biofilm formation was found to be thermostable, a property shared by pyocyanin [Denning *et al*., 1998]. This compound can further be analysed and assayed in order to study inhibition of Candidal biofilm formation in vivo, too and can pave a way for development of new antifungal agents inhibiting biofilm formation by these yeast pathogens. Thus new anti-biofilm compounds can be synthesized.

CONCLUSION

Pyocyanin can be utilised in vivo to inhibit device-associated biofilm formation by these pathogens.

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Table 1: Optical Density reading of *C. albicans* and *C. tropicalis* in YPD, heated and unheated filtrate

	In YPD	**In unheated filtrate**	**in heated filtrate**
Optical DensityO.D.) of *C. albicans* at 450 nm	3.6	0.12	0.188
Optical Density(O.D.) of *C. tropicalis* at 450 nm	3.43	0.14	0.165

(p value <0.05 by Z test in both cases)

Table 2: Optical density reading of *Candida albicans* and *C. tropicaiis* in YPD and Pyocyanin

	In YPD	**In Pyocyanin**
Optical Density (O.D.) of *C. albicans* at 450 nm	1.515	0.081
Optical Density (O.D.) of C. *tropicalis* at 450 nm	0.939	0.080

(p value: <0.05 by Z test in both cases)