Indian J. Microbiol. (December 2008) 48:438–444

ORIGINAL ARTICLE

Biofilm formation by *Candida albicans* isolated from intrauterine devices

Priyanka Lal · Vishnu Agarwal · Parul Pruthi · Ben M. J. Pereira · M. R. Kural · Vikas Pruthi

Received: 4 August 2007 / Accepted: 21 January 2008

Abstract Our survey revealed that infected intrauterine devices (IUDs) recovered from patients suffering from reproductive tract infections (RTIs) were tainted with *Candida* biofilm composed of a single or multiple species. Scanning electron microscopy (SEM) analysis of *C. albicans* biofilm topography showed that it consists of a dense network of mono- or multilayer of cells embedded within the matrix of extracellular polymeric substances (EPS). Confocal scanning laser microscopy (CSLM) and atomic force microscopy (AFM) images depicted that *C. albicans* biofilms have a highly heterogeneous architecture composed of cellular and noncellular elements with EPS distributed in the cell–surface periphery or at cell–cell interface. Biochemical analysis showed that EPS produced by *C. albicans* biofilm contained significantly reduced total carbohydrate (40%), protein (5%) and enhanced amount of hexosamine (4%) in contrast to its planktonic counterparts. The *in vitro* activity of antifungal agents amphotericin B, nystatin, fluconazole and chlorhexidine against pre-formed *C. albicans* biofilm, assessed using XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide) reduction assay revealed increased resistance of these infectious biofilm (50% reduction in metabolic activity at a concentration of 8, 16, 64, 128 μg/ml respectively) in comparison to its planktonic form.

P. Lal[1] · V. Agarwal[1] · P. Pruthi[2] · B. M. J. Pereira[1] · M. R. Kural[3] · V. Pruthi[1] (✉)
[1]Department of Biotechnology,
Indian Institute of Technology Roorkee, Roorkee - 247 667, India
[2]High Altitude Plant Physiology Research Centre, HNB Garhwal University, Post Box - 14, Srinagar, Garhwal, Uttaranchal, India
[3]Chief Medical Officer, Institute Hospital,
Indian Institute of Technology Roorkee, Roorkee - 247 667, India

E-mail: vikaspruthi@yahoo.com, vikasfbs@iitr.ernet.in

Keywords Biofilm · *Candida albicans* · EPS · XTT

Introduction

The safety, effectiveness and acceptability of the currently available intrauterine devices (IUDs) have ranked it to be the most popular reversible contraceptive method used by millions of women worldwide [1]. The tiny devices that are fitted inside the uterus are now available in several shapes and sizes ranging from Dalkon Shield, Lippes Loop, Margulies Spiral to copper IUDs (T Cu-380A, T Cu-220C, Nova 7 and ML Cu-375). Besides these, IUDs such as Progestasert and LNG-20, which constantly release small amounts of steroids into the uterus, are also available in the market [2]. On insertion, IUDs stimulate a pronounced inflammatory reaction or foreign body response in the uterus, which is responsible for the effective contraception. Despite being cost-effective in controlling birth rate and providing a high degree of sexual satisfaction to its users, IUDs are still having dark cloud hung over them. Researchers have shown that IUD use increases the risk of pelvic inflammatory diseases, and leads to heavier periods, menstrual cramps and complications associated with colonization of microbes on these implanted IUDs [3]. The majority of the infected IUD threads, made up of polyvinylchloride (PVC) material, were found to be colonized with *Candida* spp. Importantly, *Candida* has been shown to be the third most commonly isolated bloodstream pathogen from US hospitals, now surpassing gram-negative rods in frequency [4]. Moreover, the increase in *Candida* infections in the past decades has almost paralleled the increase and widespread use of a broad range of medical implant devices, mainly in populations with impaired host defenses. The objective of the present

study is to explore the nature of biofilm formed during *C. albicans* infection in gynecologic infections among women with implanted IUDs and to investigate *C. albicans* matrix polymers that might act as a fence to the diffusion of antifungal agents thereby restricting the accessibility of these to penetrate deep into the architecture of the biofilm.

Materials and methods

Materials and microorganism

Infected IUDs were collected from local nursing homes and from the hospital at the Indian Institute of Technology, Roorkee, India, from patients suffering from reproductive tract infection (RTI) under aseptic conditions and brought to the laboratory for investigation as described earlier [5]. Selection and simultaneous identification of *C. albicans* isolates from infected IUDs was done using Hichrom Candida agar, purchased as a powdered medium from Himedia Chemicals, India. The medium was prepared according to manufacturer's instructions.

Culture conditions

C. albicans isolates obtained above were tested for their biofilm-forming ability at different time intervals (8, 16, 24, 32, 40, 48, 56, 64 and 72 h). The cultures were grown on yeast peptone dextrose (YEPD) broth medium containing 20 g/l peptone, 10 g/l yeast extract and 20 g/l dextrose and incubated for 48 h at 35°C with agitation (120 rpm).

Biofilm quantification

Biofilm quantification was performed using modified 2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-5-tetrazolium-carboxanilide (XTT) reduction assay on capped polypropylene tubes [6]. Briefly, 1 cm^2 pieces of PVC were dipped in 1 ml of culture with 5×10^8 cfu/ml of 48 h grown *C. albicans* and placed for 90 min of adhesion phase at 37°C. The PVC pieces were then washed with sterilized phosphate buffered saline (PBS) to remove loosely adherent cells. One ml of sterilized YEPD broth was added to the washed pieces and incubated at 37°C for 24 h. The biofilm thus formed was then quantified using XTT reduction assay as described below.

XTT reduction assay

XTT (Sigma, St. Louis, MO) solution (1 mg/ml in PBS) was prepared, filter sterilized through a 0.22 μm pore size filter, and stored at –70°C. Menadione (Sigma) solution (0.4 mM) was prepared and filter sterilized immediately before each assay. Prior to each assay, XTT solution was thawed and mixed with the menadione solution at a ratio of 5 to 1 by volume. The biofilms formed on PVC pieces were first washed five times with 1 ml of PBS, and then 1 ml of PBS and 60 μl of the XTT–menadione solution were added to each of the pre-washed and control tubes. The tubes were then incubated in the dark for 2 h at 37°C. Following incubation the color change in the solution was measured spectrophotometrically at 492 nm (Varian, USA).

EPS isolation

Planktonic extracellular polymeric substances (EPS) was isolated from culture supernatant of *C. albicans* using the acetone precipitation technique [7]. Briefly, 48 h grown culture supernatant was treated with 1.5 volume of chilled acetone and centrifuged at 17000xg for 15 min. The pellet so obtained was redissolved in a small volume of distilled water and centrifuged to remove any insoluble material. Water soluble polymer was reprecipitated with 2 volume of chilled acetone for 2 h at 4ºC. The precipitate was finally washed with ether and evaporated to dryness in warm air current before being weighed. The percentage yield was calculated as dry weight of EPS divided by the combined dry weight of cells and EPS. Biofilm EPS was isolated using a slight modification of the protocol given by Baillie and Douglas [6]. Briefly, biofilms grown on PVC surfaces were transferred to bottles containing 10 ml of distilled water. The bottles were sonicated for 5 min and vortexed vigorously for 1 min to disrupt the biofilms. Cell suspensions were then pooled and centrifuged. The supernatants were concentrated to one-tenth of the original volume using an Amicon DC2 hollow fibre system (Millipore Ltd, Watford, UK) and dialyzed at 4°C for 3 days against five changes (5 l each) of distilled water. The retentates were freeze-dried.

EPS analysis

Total carbohydrate content for both planktonic and biofilm EPS were analyzed quantitatively by phenol sulphuric acid method using mannose as standard [8], protein content was determined by Lowry method, phosphorus by Ames method [9], hexosamine by Blumerkrantz and Asboe-Hansen method [8] and GOD/POD kit (Excel Diagnostics, India) was used for glucose estimation.

Scanning electron microscopy

Scanning electron microscopy (SEM) was performed in accordance with the procedure given by Hawser and Douglas [10]. Briefly, *C. albicans* biofilms formed on PVC pieces

(1 cm^2) were fixed with 2.5% (v/v) glutaraldehyde in PBS for 2 h at room temperature. They were then treated with 1% (w/v) uranyl acetate for 1 h, and washed with distilled water. The samples were dehydrated in ethanol series (50%, 80%, 90% and 100%). All samples were dried to critical point by Polaron critical point drier, gold coated and viewed under a scanning electron microscope (Leo 435, England).

Confocal laser scanning microscopy

C. albicans biofilms formed on PVC surface were fixed with 2.5% glutaraldehyde in PBS for 1.5 h and visualized with fluorescent stains with 8 µM propidium iodide (PI) and fluorescein isothiocynate-concanavalin A (FITC-ConA; 50 µg/ml) in 10 mM hydroxyethylpiperazine ethanesulfonic acid (HEPES)/ 0.1 M NaCl, containing 0.1 mM Ca^2 and 0.01 mM Mn^2. Confocal laser scanning (Radiance 2100, BioRad) was performed using a Nikon microscope (objective Plan Apo 60X/1.4 oil, Japan). The excitation wavelength for PI fluorescence was 543 nm (Green He Ne laser) and fluorescence was detected through emission filter HQ 590/70 (high-quality band pass), centered at 590 nm with 70 nm bandwidth. Image processing and graph analysis was carried out with Lasersharp2000 (BioRad) and Photoshop 5.5 (Adobe Systems, San Jose, CA) was used for the final image assembly.

Atomic force microscopy

Images of biofilm sample were obtained with commercial atomic force microscopy (AFM) (NTEGRA NT-MDT, Russia). All images were collected in semi-contact mode using sharpened silicon nitride cantilevers NSG10S with spring constant about 10 N/m. The cantilevers had an amplitude range 5–15 nm, tip radius 10 nm and cone angle of 22 degree. Height and deflection images were simultaneously acquired at a scan rate of 250 kHz. Data analysis was done using NOVA software.

Antifungal susceptibility to *C. albicans* biofilm

Following biofilm formation on microtitre plate (MTP), growth medium was removed and MTP wells were washed with PBS. YPED broth (200 µl) containing different concentrations of antifungal agents were then added to each well. Antifungal concentrations used in the study ranged from 0.064–64 µg/ml for amphotericin B and nystatin while for fluconazole and chlorhexidine it ranged from 0.064–128 µg/ml to 0.064–256 µg/ml respectively. The plates were incubated for 24 h at 37°C and washed in PBS. Biofilm quantification was done by XTT reduction assay as described above.

Statistical analysis

All experiments were performed in triplicate and results were expressed as means ± standard deviations. Statistical analyses of the differences between mean values obtained for experimental groups were performed using Student's t-test. P-values of 0.05 or less were considered significant.

Results and discussion

Uterine secretions under normal conditions actively deal with microbes harboring in the vicinity of vagina and surrounding regions of the female reproductive tract. However, at times the presence of IUD provides an ideal forte for the microbes to form and boom as biofilms on these devices. Most often these microbes migrate through the cord/thread attached to check the presence of device in place. In fact, the cord/thread acts as a wick to allow microbes to travel by capillary action and enter the endometrial cavity. Among different microbes reported earlier by our group [5], the emergence of *Candida* species notably, *C. albicans*, an opportunistic pathogen in these infected IUDs are of serious concern [11]. The differential media, Hichrom Candida agar, allows selective identification of *C. albicans* (green color) based on color reaction and colony morphology with a high degree of accuracy (Fig. 1). Chromogenic agar media not only facilitates the detection and identification of *C. albicans* from mixed cultures but also provides results 24 to 48 h sooner than the standard isolation and identification procedure [12].

Fig. 1 Detection of *Candida albicans* colonies on Hichrom Candida agar.

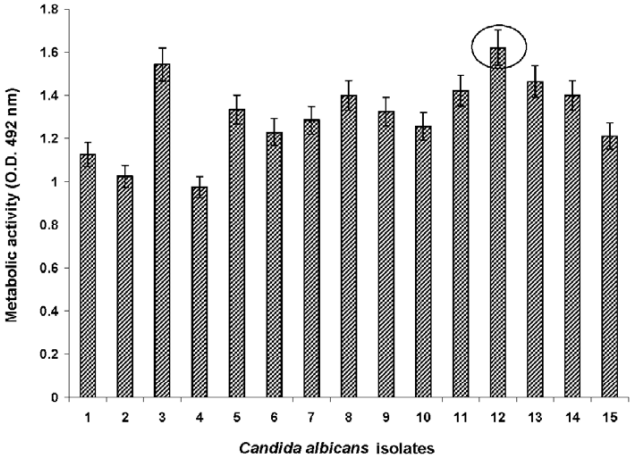

Fig. 2 Biofilm formation by *C. albicans* isolates at 48 h of incubation using XTT reduction assay. Data represent mean of ± SD of 6 independent determinations where assays were carried out in triplicate.

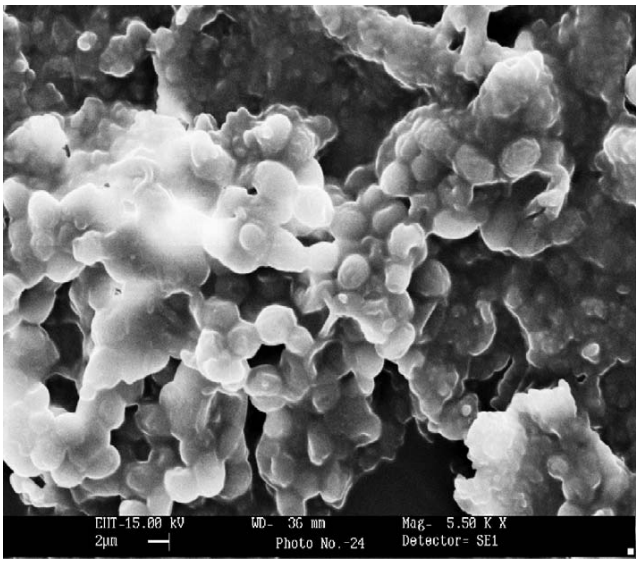

Fig. 3 Scanning electron micrograph of *Candida albicans* biofilm on PVC surface.

The tetrazolium salt XTT was used to monitor *C. albicans* biofilm formation recovered from infected IUDs by colorimetric determination. The salt is reduced by *C. albicans* mitochondrial dehydrogenase to a brown color water-soluble tetrazolium formazan product, determined spectrophotometrically (OD 492 nm). Data showed that out of the 15 *C. albicans* isolates screened for biofilm formation at different time periods (8–72 h), maximum biofilm formation was achieved by isolate number 12 at 48 h revealing its strongest ability to form biofilm compared to all other isolates (Fig. 2).

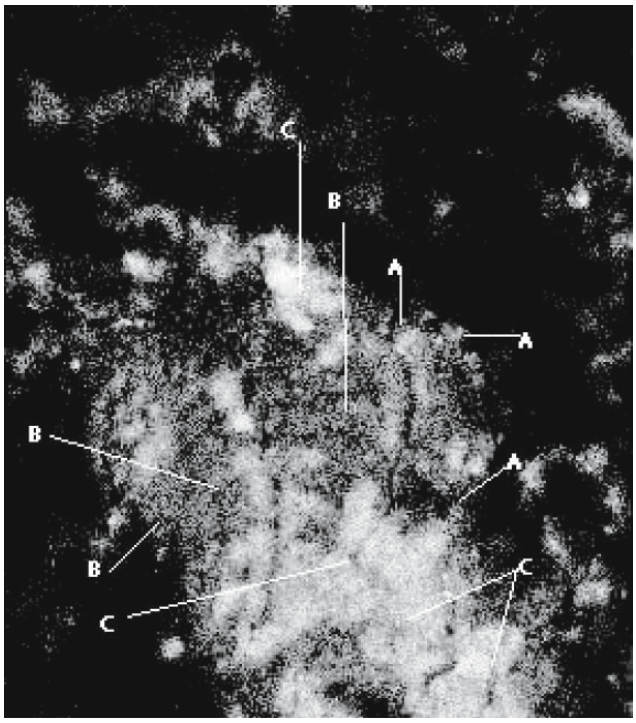

Fig. 4 CLSM image of *Candida albicans* biofilm. Figure showing PI stained dead cells (arrow A), FITC-Con A stained polysaccharides (arrow B) and formation of capsular components due to overlapping of FITC-ConA and PI images (arrow C).

SEM images of *C. albicans* biofilms adherent to PVC surfaces are shown in Figure 3. Images revealed that *C. albicans* biofilms were composed of a dense layer of yeast cells adherent to the PVC material encased in a thick matrix of extracellular polymers.

The heterogeneous structure of *C. albicans* biofilm was analyzed by CLSM using fluorescent stains FITC-ConA and PI. FITC-ConA is known to selectively bind to the mannose and glucose residues of biofilm polysaccharides [13]. PI is a fluorescent nucleic acid stain that enters dead/nonviable by binding to double-stranded nucleic acids through intercalation between base pairs with no preference to purine or pyrimidine base pairs [14]. Figure 4 shows intense green fluorescence (arrow B) resulting from ConA binding to polysaccharides of *C. albicans* while PI penetrates only cells with damaged membranes and stains the cells red (arrow A). Thus, areas of red fluorescence (arrow A) represent dead cells and green fluorescence (arrow B) indicates dense network of polysaccharides. The yellow color (arrow C) formed as a result of overlapping of red and green images depicts production of exopolysaccharides as capsular components. These analyses revealed a highly heterogeneous architecture of mature *C. albicans* biofilms in terms of the distribution of fungal cells and EPS.

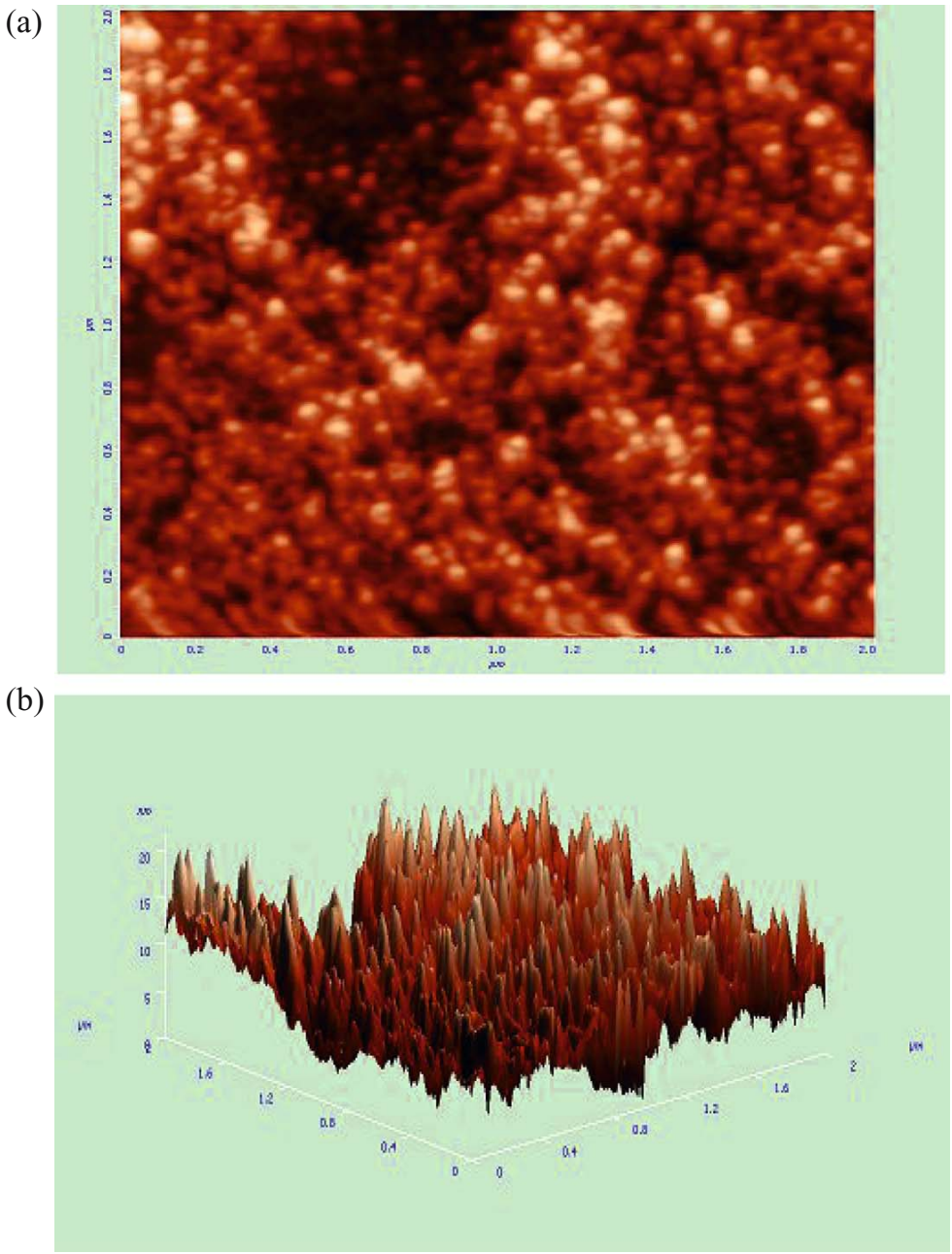

Fig. 5 **(a)** Atomic force microscopic images of *C. albicans* biofilm on PVC surface. **(b)** 3-D image of *C. albicans* biofilm depicting the ridges formation having Z (height) scale 30 nm and X-Y scales 2 µm.

Figure 5a shows AFM image of *C. albicans* biofilm on PVC surface in semi-contact mode. Image depicted that *Candida* cells were embedded within a sticky layer of EPS distributed around the cell surface, particularly at the cell–substrate periphery. Figure 5b illustrates 3D image of *C. albicans* biofilm revealing a thin colorless layer of EPS surrounding the biofilm residing *Candida* cells. The 3-D image significantly gives a better image resolution sensing height and texture variations of biofilm on the PVC surface in contrast to optical microscopy. The biofilm cells were seen as ridges in the 3-D image and were typically 5–30 nm above the lowest point. Our results are in agreement with the findings of Touhami *et al* [15], who showed that these ridges may be due to variable production of EPS, such that EPS is sometimes produced in such a great quantity that it protrudes around the cells and become more pronounced after drying of the biofilm [16]. Recently AFM studies conducted by Oh *et al* [17], have shown similar pattern of biofilm structure in the case of *E. coli* and also suggested that this sticky EPS accumulates in the cell surface and

Table 1 Percentage composition of planktonic and biofilm EPS obtained from *C. albicans*. Results represent mean of ± SD of 6 independent determinations where assays were carried out in triplicate

C. albicans	Total carbohydrate	Phosphorus	Protein	Glucose	Hexosamine
Biofilm	40 ± 8.5	0.7 ± 0.2	5.0 ± 1.2	16 ± 3.4	4.0 ± 1.3
Planktonic	86 ± 6.5	0.4 ± 0.1	9.0 ± 1.5	5.0 ± 1.6	0.6 ± 0.2

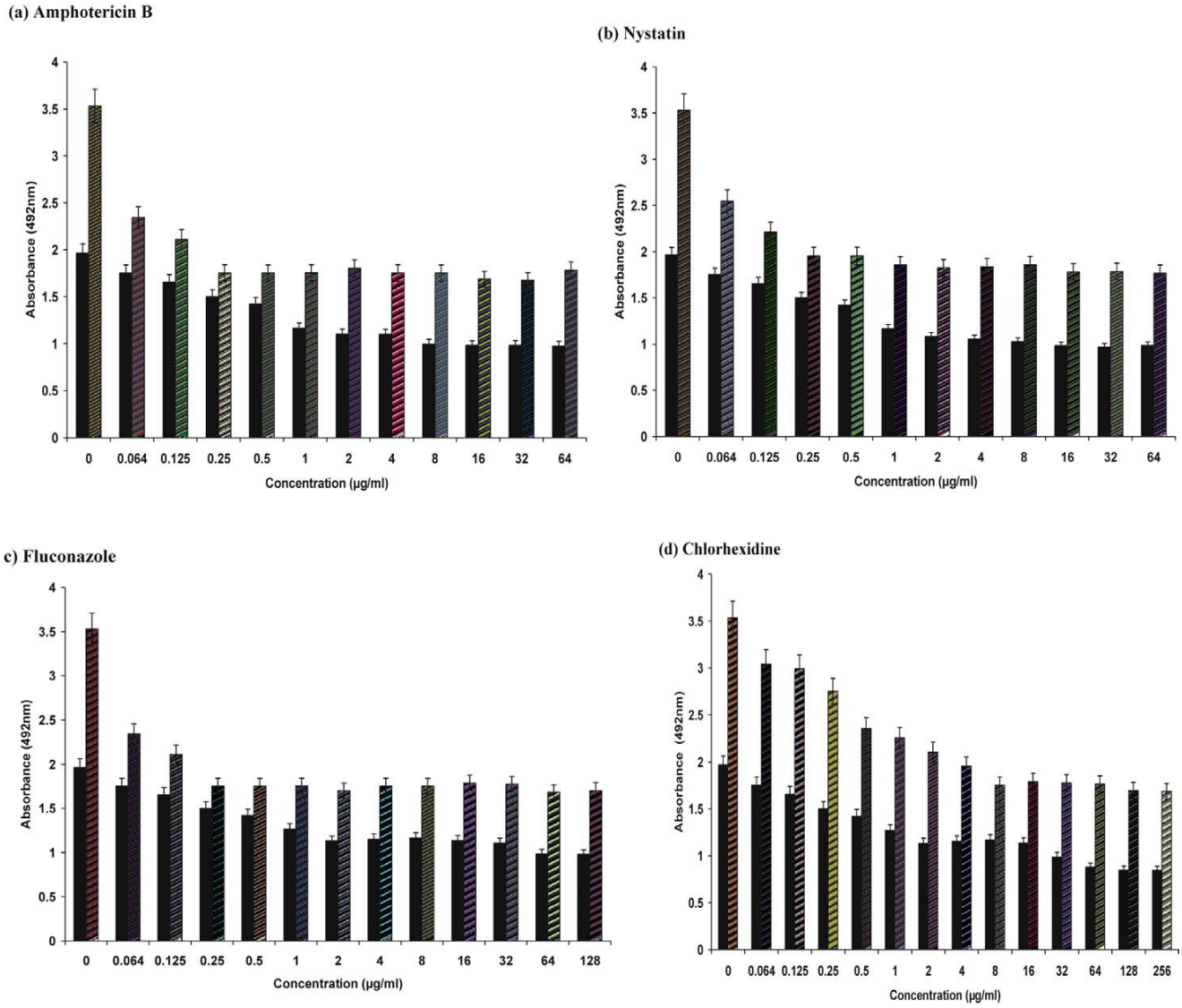

Fig. 6 Susceptibility of *Candida albicans* for ■ biofilm and ⊡ planktonic to different concentrations of antifungals. Metabolic activity was normalized to control without drugs which was taken as 100%. Results represent mean of ± SD of 6 independent determinations where assays were carried out in triplicate.

enhances aggregation of microbial cells, leading to the formation of biofilms.

The composition of EPS surrounding the planktonic as well as biofilm forming *C. albicans* consist mainly of carbohydrate, protein, phosphorus and hexosamine (Table 1). In contrast to its planktonic counterpart *C. albicans* biofilm EPS contained significantly less carbohydrate (40%) and protein (5%). The fact that the matrix of EPS might act as a barrier to the diffusion of antifungal agents and so limit the access of drug to organism deep in the biofilm has been explored earlier [7, 18]. Baillie and Douglas [6], reported that synthesis of *C. albicans* biofilm

on biocompatible material is highly dependent on the conditions of incubation.

Our investigation to test *in vitro* activity of antifungal agents revealed increased susceptibility of planktonic form in comparison to the biofilm form of *C. albicans.* Data showed 50% reduction in metabolic activity for planktonic cells at a concentration of 0.25, 1.0, 0.25, 8 μg/ml for amphotericin B, nystatin, fluconazole and chlorhexidine compared to 8, 16, 64, 128 μg/ml for biofilm respectively (Fig. 6). The results are in agreement with the observations of Hawser and Douglas [10] (1994), who showed that *C. albicans,* growing in catheter-associated biofilms was resistant to antifungals. These results strongly support the contention that patients with RTIs are more prone to acquire *C. albicans* biofilm-associated infection because of higher antifungal resistance. The phenotypic changes resulting from nutrient limitation, EPS production and differential expression of drug resistance gene can be considered as the possible mechanism of antifungal resistance among biofilm forming *Candida* species on IUDs [3]. A better understanding of *Candida* biofilms may lead to the development of novel therapeutic approaches for the treatment of fungal infections due to implants especially in gynecologic infections among women with implanted IUDs.

Acknowledgements This work was financially supported by the Council of Scientific and Industrial Research (CSIR) and Department of Biotechnology (DBT), Government of India. Authors are thankful to International Centre for Genetic Engineering and Biotechnology (ICGEB), India for providing confocal microscopy facility, which is funded by senior fellowship Wellcome Trust UK.

References

1. Hsiao YC and Sung SH (2003) Married women's satisfaction with their choice of contraception. J Nurs Res 11:119–128
2. Arancibia V, Pena C, Allen HE and Lagos G (2003) Characterization of copper in uterine fluids of patients who use the copper T-380A intrauterine device. Clin Chima Acta 332: 69–78
3. Pál Z, Urbán E, Dósa E, Pál A and Nagy E (2005) Biofilm formation on intrauterine devices in relation to duration of use. J Med Microbiol 54:1199–1203
4. Ramage G, Vandewalle K, Wickes BL and López–Ribot JL (2001) Characteristics of biofilm formation by *Candida albicans.* Rev Iberoam Micol 18:163–170
5. Pruthi V, Al-Janabi A and Pereira BJ (2003) Characterization of biofilm formed on intrauterine devices. Indian J Med Microbiol 21:161–165
6. Baillie GS and Douglas LJ (2000) Matrix polymers of *Candida* biofilms and their possible role in biofilm resistance to antifungal agents. J Antimicrob Chemother 46: 397–403
7. McCourtie J and Douglas LJ (1985) Extracellular polymer of *Candida albicans*: isolation, analysis and role in adhesion. J Gen Microbiol 131:495–503
8. Chaplin MF and Kennedy JF (1986) Carbohydrate analysis IRL press, Washington DC, USA
9. Ames BN (1966) Assay of inorganic phosphate, total phosphate and phosphatases. In: Neufeld EF and Ginsburg V (eds). Methods in enzymology Vol. 8: Complex carbohydrates. Academic Press, New York, pp. 115–118
10. Hawser SP and Douglas LJ (1994) Biofilm formation by *Candida* species on the surface of catheter materials *in vitro*. Infect Immun 62:915–921
11. Ramage G, Saville SP, Thomas DP and López-Ribot JL (2005) *Candida* biofilms: an update. Eukaryot Cell 4: 633–638
12. Pfaller MA, Houston A and Coffmann S (1996) Application of CHROMagar *Candida* for rapid screening of clinical specimens for *Candida albicans*, *Candida tropicalis*, *Candida krusei*, and *Candida* (*Torulopsis*) *glabrata.* J Clin Microbiol 34(1):58–61
13. Takenaka S, Iwaku M and Hoshino E (2001) Artificial *Pseudomonas aeruginosa* biofilms and confocal laser scanning microscopic analysis. J Infect Chemother 7: 87–93
14. http://en.wikipedia.org/wiki/Propidium_iodide
15. Touhami A, Jericho MH, Boyd JM and Beveridge TJ (2006) Nanoscale characterization and determination of adhesion forces of *Pseudomonas aeruginosa* pili by using atomic force microscopy. J Bacteriol 188(2): 370–377
16. Emerson RJ and Camesano TA (2004) Nanoscale investigation of pathogenic microbial adhesion to a biomaterial. Appl Environ Microbiol 70(10):6012–6022
17. Oh YJ, Jo W, Yang Y and Park S (2007) Influence of culture conditions on *Escherichia coli* O157:H7 biofilm formation by atomic force microscopy. Ultramicroscopy 107(10–11): 869–874
18. Hawser SP, Baillie GS and Douglas LJ (1998) Production of extracellular matrix by *Candida albicans* biofilms. J Med Microbiol 47:253–256