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FULL PAPER

Biofilm formation by *Candida albicans* on various prosthetic materials and its fluconazole sensitivity: a kinetic study

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Abstract *Candida albicans* has the ability to colonize various materials used in prostheses. In this report, we have studied the kinetics of biofilm formation on prosthetic materials and their susceptibility to fluconazole at various stages of development. Results indicated that *C. albicans* efficiently adheres to and colonizes polystyrene, polyvinylchloride, silicon, and polycarbonate surfaces. *Candida albicans* biofilm formation was observed to be both strain- and substrate dependent. Adhesion of cells to solid substrates was found sufficient to induce fluconazole resistance. Drug susceptibility at different stages of biofilm growth showed that *Candida* biofilms on these substrates are highly resistant to fluconazole. The study focuses on the limitations of fluconazole to combat biofilm-related infections and emphasizes the need for better therapeutic strategies against prosthesis-associated *C. albicans* infections.

Keywords Biofilm · *Candida albicans* · Drug resistance · Fluconazole · Medical devices

Introduction

In nature, most microorganisms live as surface-associated communities in the form of biofilms (Donlan and Costerton 2002). *Candida albicans*, a commensal of humans, is no exception. It colonizes host tissues as well as the surfaces of various prosthetic devices implanted in a patient's body (Chandra et al. 2001a). *Candida albicans* is reported to form biofilms on urinary catheters, venous catheters, denture materials, central nervous system prostheses, artificial heart valves, joint prostheses, contact lenses, penile implants, and intrauterine devices. These prostheses are made of a variety of materials such as polyvinylchloride (PVC), polymethyl-methacrylate, silicon, polyurethane, polycarbonate, and polypropylene (Kojic and Darouiche 2004; Ramage et al. 2006). A large population of immunocompromised patients as well as surgical advances in medical technology have resulted in frequent use of prosthetic devices, paralleled by increased incidence of bloodstream infections as well as device-related biofilm infections by opportunistic *C. albicans* (Douglas 2002, 2003; Andes et al. 2004).

A characteristic feature of *C. albicans* biofilms is resistance to most of the available antifungal drugs, including widely prescribed azoles such as fluconazole. Susceptibility studies have revealed that biofilms formed by *C. albicans* may be up to 2,000 times more resistant to antifungal drugs than their planktonic counterparts (Jabra-Rizk et al. 2004; Baillie and Douglas 2000; Chandra et al. 2001b). Moreover, biofilms may act as reservoirs and release cells into the body. Even if free cells in the body are removed by antibiotic treatment, the reservoir remains unaffected, to cause reinfection (LaFleur et al. 2006; Khot et al. 2006). The only option in such cases is removal or replacement of the colonized devices. Hence, biofilm-associated *Candida* infections are difficult to treat and considered as a real threat to an immunocompromised population (Jabra-Rizk et al. 2004).

Various reasons have been proposed for the antifungal resistance of *C. albicans* biofilms (Lewis 2001; Mukherjee et al. 2003; Ramage et al. 2002); however, no single reason could explain it. A recent report has shown that

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extracellular glucan is involved in sequestering of antifungals, thereby making biofilms resistant to their action (Nett et al. 2010). Contact of a surface is known to induce a cell integrity pathway that plays an important role in the development of *C. albicans* biofilms and drug resistance (Kumamoto and Vinces 2005). Differential gene expression after adhesion on the solid substrate results in activation of efflux pumps, seizing of metabolic rate, and increased production of the extrapolymeric matrix with glucan, and may contribute to the drug resistance in biofilms (Nett et al. 2010; Kumamoto 2002). The physical and chemical nature of the contact surface and specific properties of cells are important factors known to influence adhesion as well as biofilm formation (Douglas 2002; Jabra-Rizk et al. 2004; Raut et al. 2010).

Most of the previous studies have discussed *Candida* biofilms on acrylic and silicon. In available reports, polystyrene plates have been used as a model to study adhesion and biofilm formation (Ramage et al. 2001; Imbert et al. 2003; Soustre et al. 2004; Jain et al. 2007). An in vitro model for drug susceptibility of *Candida* biofilms was standardized on 96-well polystyrene plates (Ramage et al. 2001). Also, resistance to antifungal drugs by biofilms on these surfaces was studied by different workers (Chandra et al. 2001b; Kuhn et al. 2002). Comparative studies of biofilm formation by different strains of *C. albicans* on various substrates and their drug susceptibility at different stages of development are important. The aim of the present work was to study whether variation in physical and chemical properties of the substrate and differences in the strains affect biofilm formation and thus influence the drug sensitivity of *C. albicans*. Here, we report in vitro kinetics of *C. albicans* biofilm formation on four prosthetic materials by a standard strain and two clinical isolates. Also, fluconazole susceptibility at different time points of biofilm development is discussed.

Materials and methods

Cultures

A standard strain of *C. albicans*, ATCC-90028, was obtained from the Institute of Microbial Technology (IMTECH), Chandigarh, India. Clinical isolates SRTCC-06 and SRTCC-11 were obtained from the School of Life Sciences, SRTM University, Nanded. Clinical isolates were obtained from patients suspected to have *Candida* infections. *C. albicans* SRTCC-06 was isolated from feces of a patient admitted to the tertiary care hospital for treatment of diarrhea. Another isolate, SRTCC-11, was isolated from a patient diagnosed with vaginitis. All the cultures were maintained on yeast peptone dextrose (yeast extract 1%, peptone 2%, dextrose 2%, agar 2%; YPD, HiMedia, Mumbai, India) agar slants at 4°C.

Medium and culture conditions

A single colony from YPD agar was inoculated into 50 ml YPD broth in a 250-ml Erlenmeyer flask. The flasks were incubated at 30°C for 24 h at 100 rpm on an orbital shaking incubator. Cells were harvested by centrifugation at 2,000 *g* and washed thrice with 0.1 M phosphate-buffered saline (PBS; HiMedia), pH 7.4. Cells were counted with a hemocytometer, and the stock cell suspension was used to obtain the required cell density (either 1×10^7 cells/ml or 1×10^3 cells/ml) for further experiments.

Susceptibility of planktonic cells to fluconazole

The susceptibility study was carried out by the standard methodology, M 27 A, as per Clinical and Laboratory Standards Institute (CLSI) guidelines (Richter et al. 2005). Various concentrations of fluconazole were prepared in the wells of the microplates by double dilution. Drug concentrations in the range 2–128 μg/ml (Forcan, Cipla, India) were prepared in RPMI-1640 medium with L-glutamine, without sodium bicarbonate, buffered with 165 mM MOPS, pH 7.0 (RPMI-1640 and MOPS, HiMedia Laboratories). Wells without the drug served as the control. Each well contained an inoculum of cell density 1×10^3 cells/ml. Plates were incubated at 37°C for 48 h and read spectrophotometrically at 620 nm using a microplate reader (Multiskan EX, Thermo Electronics, USA). The lowest concentration of fluconazole that caused 50% reduction in the absorbance compared to that of the control was considered as minimum inhibitory concentration (MIC). Standardized drug susceptibility protocols by CLSI have established breakpoints for fluconazole sensitivity: strains having MIC $\leq$8 μg/ml are considered sensitive; those having16–32 μg/ml are dose-dependently susceptible; cells requiring $\geq$64 μg/ml fluconazole for inhibition of growth are considered resistant (Rex et al. 2001).

Substrates used for biofilm formation

Disks of uniform size (diameter, 5.5 mm) were cut aseptically with an autoclaved punch from the test materials for biofilm formation: polyvinyl chloride (PVC) (endotracheal tube, 9.5 mm; Portex, Keene, NH, USA), polycarbonate (Hindustan Syringes and Medical Devices, Faridabad, India), and polystyrene (96-well microplates; Tarson, India). Most materials were provided sterile by the manufacturers; only the silicon disks (Airway, size 4; Anesthetics India, Mumbai, India) were sterilized by moist heat as per the manufacturer's recommendations.

Biofilm formation

The disks cut from various materials were placed in 96-well microplates; 100 µl cell suspension (1×10^7 cells/ml in PBS) was put into each well. Plates were incubated at 37°C for the adhesion phase of 90 min. Disks were washed twice with PBS to remove nonadhered cells and transferred to a fresh plate containing 200 µl RPMI-1640 medium, pH 7.0 (with L-glutamine, without sodium bicarbonate, buffered with 165 mM MOPS). Plates were incubated at 37°C with 100 rpm in an orbital shaker for 0, 6, 12, 24, or 48 h.

Fluconazole susceptibility of biofilms

The susceptibility study was carried out as per the standard methodology with little modification (Ramage et al. 2001). Briefly, various concentrations (2–128 µg/ml) of fluconazole (Forcan; Cipla) were prepared in RPMI-1640 medium. RPMI-1640 medium with varying concentrations of fluconazole was added to the wells containing disks at various time points of biofilm development. Wells without fluconazole served as control. Plates were incubated at 37°C at 100 rpm for 48 h and then washed with PBS to release unattached cells. The effect of fluconazole on biofilm growth was analyzed with the XTT-tetrazolium reduction assay.

Biofilm quantitation by XTT assay

Biofilm formation as well as their fluconazole susceptibility on different materials was quantitated using the XTT [i.e., 2,3-bis (2-methoxy-4-nitro-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide] (Sigma-Aldrich, India) reduction assay (Ramage et al. 2001). The XTT solution was prepared by mixing 1 mg/ml XTT salt in PBS and stored at –20°C. Before use, menadione solution prepared in acetone (Sigma-Aldrich) was added to XTT to a final concentration of 4 µM (Silva et al. 2008). The disks with biofilms were washed with PBS and incubated for 5 h in 100 µl XTT-menadione solution in the dark at 37°C at 100 rpm. Color formation by the water-soluble formazan product was measured at 450 nm using a microplate reader (Multiskan EX; Thermo Electronics). Disks without biofilms served as a control. MIC was defined as the lowest concentration of fluconazole at which 50% reduction in optical density [i.e., 50% reduction in relative metabolic activity (RMA)] compared to control was achieved (Chandra et al. 2001a; Ramage et al. 2001).

Microscopic analysis of biofilms

Biofilms were observed under an inverted light microscope (Metzer, India). Photographs were taken by a Labomed microphotography system (Labomed Korntal, Germany).

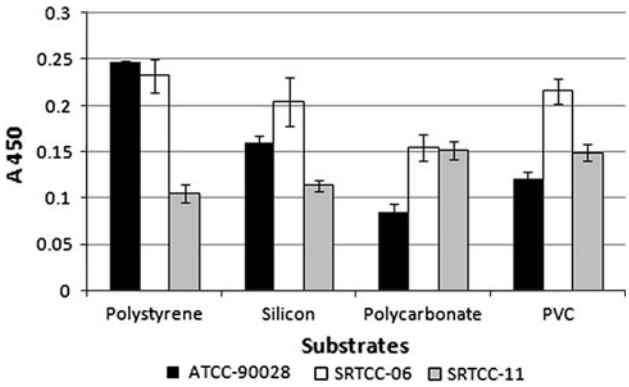

Fig. 1 Adhesion of three strains of *Candida albicans* on four substrates (*left to right*): polystyrene, silicon, polycarbonate, and polyvinyl chloride (*PVC*). Density of adhered cells was analyzed by absorbance (A_{450}) of colored end product in the XTT-metabolic assay

For scanning electron microscopy (SEM), samples were fixed in 2.5% glutaraldehyde in 0.1 mol l^{-1} phosphate buffer (pH 7.2) for 24 h at 4°C. Samples were post-fixed in 2% aqueous solution of osmium tetraoxide for 4 h, then dehydrated in a series of graded alcohols and finally dried to a critical drying point with a Critical Point Dryer unit. The samples were mounted over stubs, and gold coating was performed using an automated gold coater (model JOEL JFC-1600) for 3 min. Photographs were taken under a scanning electron microscope (model JOEL-JSM 5600).

Statistical analysis

All the experiments were performed with three strains and four substrates. Values given are the mean of triplicate observations with standard deviations indicated in parentheses. Mean biofilm formation values and values of fluconazole susceptibility were compared by two-way analysis of variance (ANOVA). *P* at the 1% level was considered significant.

Results and discussion

Different properties of cells and substrates are supposed to influence adhesion and biofilm formation (Jain et al. 2007; Xiang et al. 2004). The aim of this study was to analyze substrate and strain variation in biofilm formation and fluconazole susceptibility. The results of our work are significant as the effects of two variables (strains and substrates) on adhesion, biofilm development as well as the fluconazole sensitivity of *C. albicans* biofilms are interpreted in a single study.

Adherence varied with strains and surfaces

As measured by RMA, the adhesion of the three *C. albicans* strains on the four substrates varied significantly

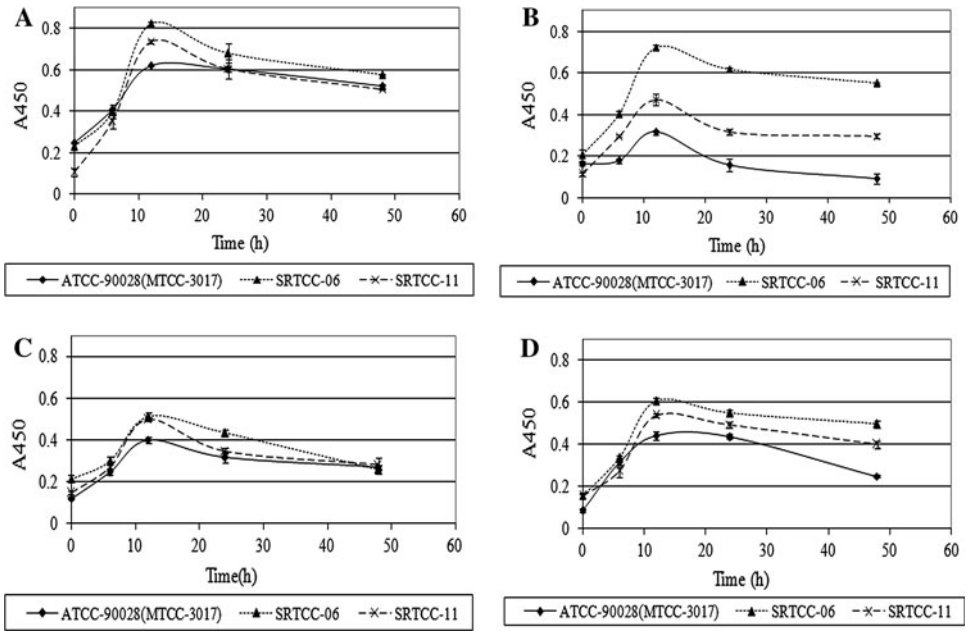

Fig. 2 Kinetics of biofilm formation by three *C. albicans* strains on four substrates: polystyrene (**a**), silicon (**b**), polyvinyl chloride (PVC) (**c**), and polycarbonate (**d**). Biofilm growth at each time point was analyzed by XTT-metabolic assay

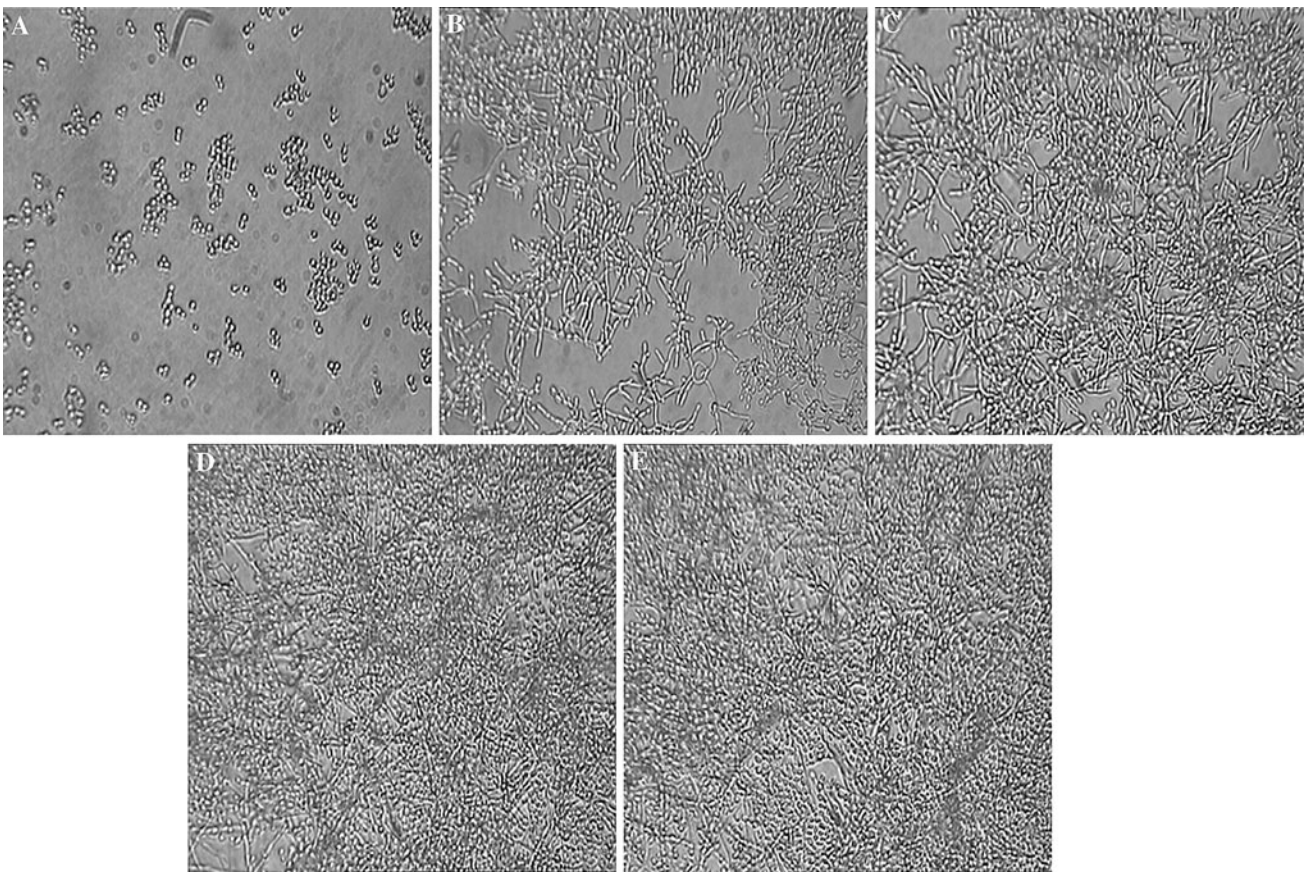

Fig. 3 Light micrographs of *C. albicans* (ATCC-90028) biofilm development on polystyrene: 0 h (**a**), 6 h (**b**), 12 h (**c**), 24 h (**d**), and 48 h (**e**). Various phases such as adhesion, yeast to hyphae conversion, and network of microcolonies lead to formation of a dense network of yeast and filamentous forms in mature biofilms. ×200

($P < 0.01$). Statistical analysis concluded that, in general, variation in the strains ($P < 0.01$) has a more pronounced effect on adhesion than that of the substrates. Polystyrene supported the highest adhesion ($P < 0.01$), followed by silicon, PVC, and polycarbonate (Fig. 1). Among the strains, SRTCC 06 showed more adhesion compared to the other two, indicating that ability to adhere to prosthetic devices also depends on the properties of the cells.

Kinetics of biofilm formation

Candida albicans readily developed biofilms on various substrates used in prosthetic devices. Monitoring by XTT assay and microscopy revealed that biofilm development by different strains on various substrates proceeds through similar stages. The initial adhesion phase (0–2 h) (Figs. 2a, 3a) was followed by dimorphic switching (2–6 h) (Figs. 2b, 3b). In the next 6 h, the surface of the substrate was colonized densely, and a multiple layer biofilm was formed by 12–24 h (Figs. 2c, d, 3c, d). Biofilm growth started declining after 24 h (as indicated by XTT metabolic activity) (Fig. 2). Biofilms formed by different strains on various substrates were similar qualitatively (as observed by light microscopy and SEM) (Fig. 4). However, the extent of the biofilm at various stages of growth was significantly ($P < 0.01$) influenced by properties of the strains as well as the substrate. In general, compared to the standard strain (ATCC-90028), the two clinical isolates of *C. albicans* were efficient biofilm formers (Fig. 2). For example, biofilm formation on silicon by three strains decreased in the order SRTCC 06 > SRTCC 11 > ATCC 90028 (Fig. 2b). Among the substrates, the trend for the extent of biofilm formation was polystyrene > polycarbonate > PVC > silicon. Various properties including roughness of the substrates and surface free energy may influence adhesion and biofilm development (Raut et al. 2010; El-Azizi and Khardori 1999). However, recent reports have shown that roughness and free energy of the surface did not affect *C. albicans* biofilm formation; instead, the characteristic properties of strains significantly influenced the biofilm development (da Silva et al. 2010). Statistical analysis of our results indicated that differences in strains contributed more to variation in biofilm growth than differences of the substrates. Interestingly, adhesion of cells to a surface did not always result in better biofilms, which suggested that the density of adhered cells was not a deciding factor but that the type of strain and substrate influenced the biofilm development prominently. In vivo,

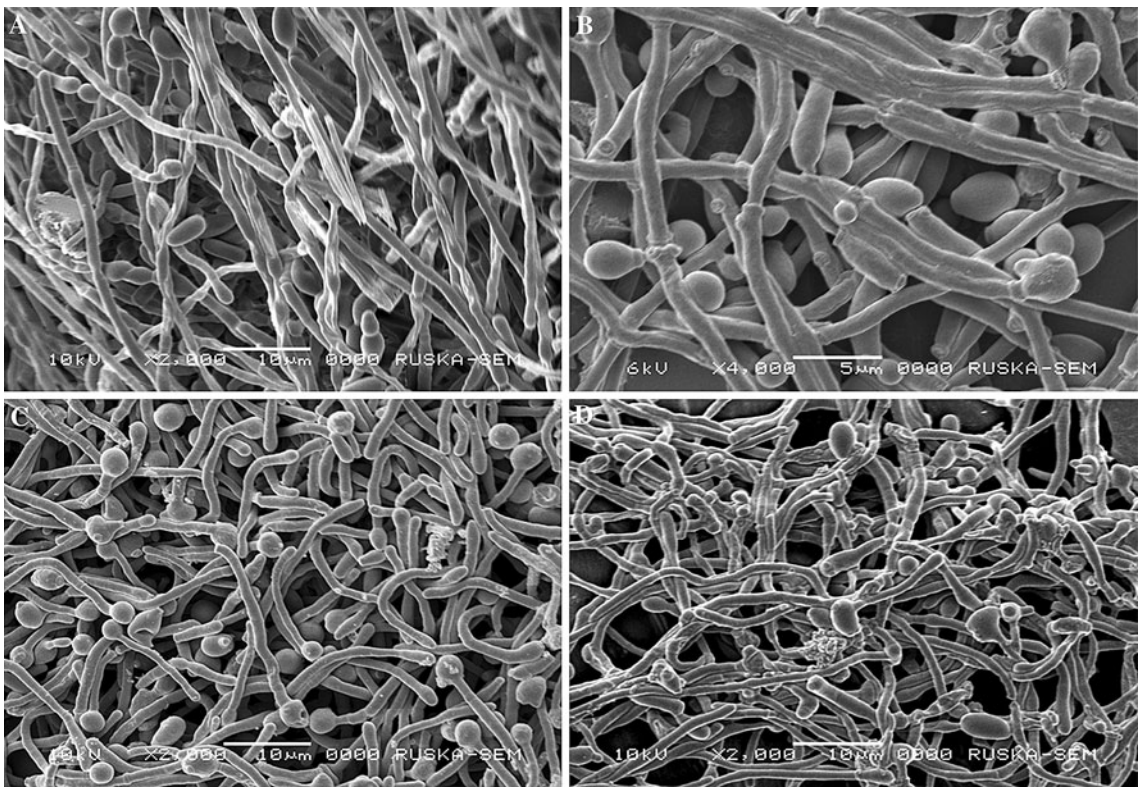

Fig. 4 Scanning electron micrographs (SEM) of 24-h-old biofilms formed by *C. albicans* (ATCC-90028) on four surfaces: polystyrene (**a**), silicon (**b**), polyvinyl chloride (PVC) (**c**), and polycarbonate (**d**). A dense network of yeast and filamentous forms is attached to the solid substrates

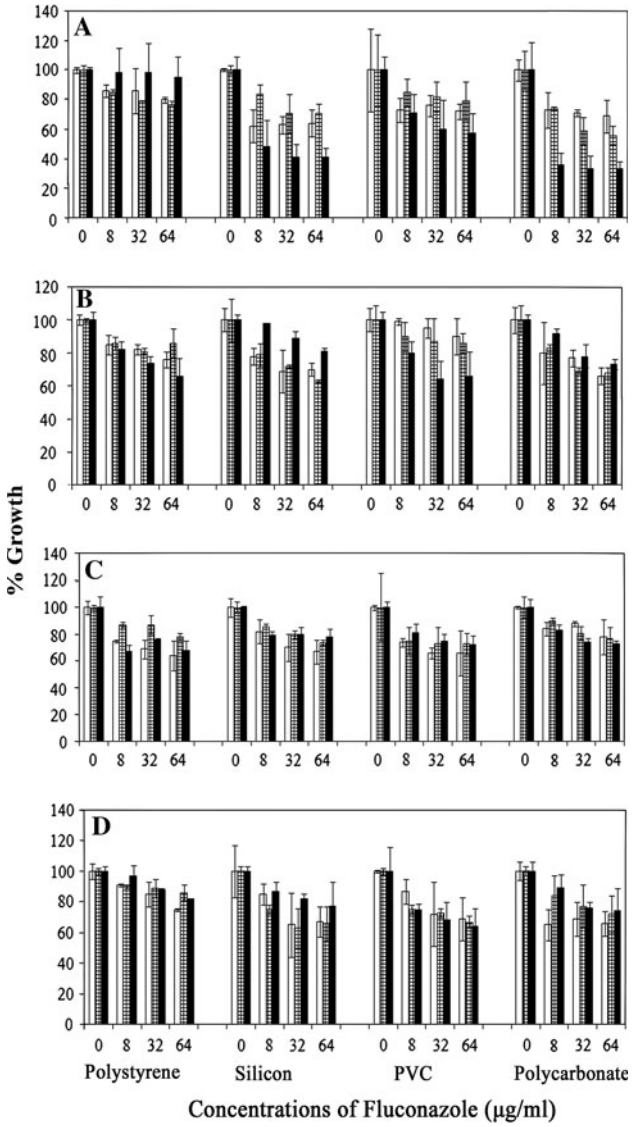

Fig. 5 Fluconazole susceptibility of three strains of *C. albicans* at various stages in biofilm development on four different substrates: 0 h (**a**), 6 h (**b**), 12 h (**c**), and 24 h (**d**). Percentage of biofilm growth calculated from the absorbance of XTT-formazan showed that biofilm growth remained unaffected in the presence of the drug fluconazole

deposition of salivary pellicle or plasma proteins on the prosthetic surfaces may be an additional factor to alter microbial colonization on prosthetic implants (Thein et al. 2007; Jin et al. 2004; Nikawa et al. 2000). Comprehensive studies including in vivo experimentation in animal models are needed to clearly understand the colonization.

Sensitivity to fluconazole

In their planktonic form of growth, all the strains studied were found sensitive to fluconazole. The MIC values for the three strains were 0.5, 1, and 2 µg/ml for ATCC-90028, SRTCC-06, and SRTCC-11, respectively. Antifungal susceptibility revealed that *C. albicans* biofilms on silicon, polystyrene, polycarbonate, and PVC were considerably resistant to fluconazole at all time points of growth (Fig. 5). In general, fluconazole sensitivity of biofilms formed by three strains on four different substrates did not vary significantly ($P > 0.05$). Interestingly, even the adhered cells were substantially resistant toward fluconazole. Addition of 2–64 µg/ml fluconazole at time point 0 (i.e., after the adhesion phase) of biofilm development on various substrates resulted in only 30% reduction in biofilms (Fig. 5a). After 6 h of biofilm development, fluconazole was totally ineffective, and even at 64 or 128 µg/ml biofilm growth remained unaffected (Fig. 5b). Then, with increasing time in biofilm development, sensitivity to fluconazole decreased. At the 24-h time point, mature *C. albicans* biofilms were completely resistant to fluconazole (Fig. 5c, d). The small decrease in biofilm growth as indicated by the decrease in metabolic activity may be because of growth in the presence of fluconazole (Gomes et al. 2011); however, the inhibition obtained was not significant.

To summarize, comparison of the kinetics of biofilm formation suggested that properties of clinical isolates and the type of prosthetic materials implanted in the patient's body may influence the risk of *C. albicans* infection. These properties may include cell-surface hydrophobicity, growth rate, and cell-surface adhesins as well as roughness of the substrate material and its chemical composition. Our study gave insight into the effect of strain and substrate variation on biofilm development, suggesting that properties specific to *C. albicans* strains may significantly contribute to variation in biofilms. Susceptibility studies at various time points in biofilm development revealed their insensitivity to the widely prescribed drug fluconazole irrespective of strain, substrate, and developmental stage. The need of better prophylaxis and therapeutic strategies against biofilm-associated *C. albicans* infections is suggested.

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