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Characterization of biofilms produced by *Candida* species isolated from tertiary care hospitals, Rawalpindi, Pakistan

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Abstract

Biofilm formation is one of the important virulence factors of *Candida* species. *Candida* species are able to form biofilm on any biotic or abiotic surface. These biofilms can cause recurrent infections as they overtake the immune responses and are resistant to most of the available antifungals. The present study was conducted to characterize the biofilm formation by clinical isolates of *Candida* obtained from a tertiary care hospital, Rawalpindi, Pakistan. The biofilms were quantified using quantification methods such as dry weight measurement and XTT reduction assays. *Candida albicans* strain SC 5314 was used as a control in each experiment. The structural organization 3D was studied by confocal laser microscopy. Additionally a well-established 96 well plate method was used to study the antifungal susceptibility of the biofilms. It was observed that all the Candida sp. form biofilm and was highly resistant to fluconazole. Caspofungin was effective against all species of the *Candida* included in the study but *Candida glabrata* showed reduced suceptibilty towards it. We also showed that biofilms formed by the *Candida glabrata* are significantly different probably due to the inability to form hyphae.

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Introduction

Candida is the member of microbiota of the gastrointestinal tract and urethral lining of healthy individuals. These commensals can become opportunistic pathogens causing diseases ranging from mucosal to severe life threatening invasive mycoses, particularly in individuals with an impaired immunity (1). *Candida* causes a high rate of mortality and morbidity in immunocompromised patients. Candidiasis is widespread in people having organ transplant, receiving chemotherapy, diabetes, low weight infants and critically ill patients. AIDS patients are also at risk (2).

Most of the resistant *Candida* infections are related to one of its important virulence factors, the formation of biofilm (3). Biofilms are the well-structured communities of yeast hyphae and pseudohyphae living together in a self-secreted exopolymeric matrix attached to any biotic and abiotic surface (4,5). The distinguishing feature of biofilms is their improved resistance to antifungals as compared to the planktonic form and to bear up to all the defenses of the immune system (6-8). These features of biofilms make its treatment very tricky and often the infection comes back again (9). For successful treatment these medical devices are generally required to be removed, which sometimes prove fatal for the patient (10).

Candida albicans biofilms have been studied extensively as compared to the non-*albicans* species (6,9,11). It has been known that *C. tropicalis*, *C. glabrata*, C. *parapsilosis* and *C. krusei* also have ability to form biofilms (12-14) but the phenomenon of biofilm formation varies among different *Candida* species. The strains of *Candida* species, which produce biofilms, are reported to be the reason of increased mortality in immunocompromised patients (15,16). Among different groups of antifungals, echinocandins have been found active against *Candida* biofilm infection (17,18).

Candida biofilms have a three dimensional heterogeneous structure having water channels interconnected with hyphae and pseudohyphae (5). The techniques like fluorescence and confocal microscopy, are used to study the gross morphology and structure, whereas XTT assay and dry weight measurement, for quantification of biofilms (6). The mitochondrial dehydrogenases of the active living yeast cells reduce the XTT to a formazan product (19). Dry weight determination allows the comparative analysis of the whole biofilms mass of a species and between different species (20).

The present study was conducted to characterize the biofilms from different Candida species using different techniques such as fluorescence and confocal scanning laser microscopy. The biofilms were also quantified by dry weight and XTT reduction assay. The antifungal susceptibility of biofilms was also determined by 96 well microtitre plate assay method.

Materials and methods

Strains

Twenty-five strains of different *Candida* sp. isolated from hospitalized patients were included in this study. The strains with their origin are given in the table 1. *Candida albicans* SC 5314 (6) biofilm producer was used as control. Candida sp. were identified by germ tube tests, colony color on Chromagar and API 20C-AUX.

Medium and culture conditions

All the *Candida* isolates were grown in yeast nitrogen base medium (YNB; Difco Laboratories, Detroit, Mich.) supplemented with 50 mM glucose, from a fresh potato dextrose agar plate for 24 hr in a shaking water bath (90 oscillation/min) at 37^{o}C. The cells were collected and washed thrice with phosphate buffer saline (PBS). A standard inoculum size (1x10^{7}) was made by counting cell in a suspension by hematocytometer.

Materials

Silicone elastomer (SE) sheets were purchased from Invotec International (FL, USA) as it is known to support biofilm formation (21). The SE sheets were cleaned by washing thrice with hand soap and water

and finally rinsed with distilled water. About 1.5cm circular disks were cut out with the help of cork borer and then autoclaved.

Biofilm formation

Silicone elastomer discs were pretreated by incubation in fetal bovine serum (FBS) (Hyclone, Utah) for 24h at 37°C on a rocker table (Bellco Glass Inc., Vineland, N.J.) in 12-well tissue culture plates (Becton Dickinson, Franklin Lakes, N.J). The rocker table ensures linear continuous flow of FBS over the surface of the SE discs. After 24h, the discs were transferred to a 12 well plate containing the standard inoculum 10^7 cells/ml for 90 min at 37°C without any rocking. After adhesion phase the discs were gently transferred to new 12 well tissue culture plate containing 4ml yeast nitrogen base (YNB) with 50 mM glucose per well for biofilm formation and were incubated for 48h at 37°C on a rocker table (for biofilm formation). All assays were carried out in triplicate.

Microscopy of Biofilms Formed by Candida sp.

Fluorescence microscopy

The gross morphology of biofilm was studied by fluorescence microscopy as described earlier (6). SE discs with mature biofilms (48h) were transferred to microscope slides and stained with 50µl of KOH and 50µl of calcofluor-white [0.05% (vol/vol)]; Sigma Chemical Co., St. Louis, Mo.) For 1 minute that fluoresces in the UV range (λ_{max}= 432 nm). Biofilms stained with calcofluor-white were examined under a fluorescence microscope (ZVS-47E microscope; Carl Zeiss, Inc., Oberkochen, Germany) and images were captured immediately.

Confocal scanning laser microscopy

Confocal microscopy was performed in order to study the architecture of mature biofilms. Fun-1(10µM) and concanavalin A-Alexa Fluor conjugate (ConA; 25µg/ml) were used together to stain the biofilms as described earlier (22)(Chandra *et al.,* 2008). The metabolically active cells convert the FUN-1TM (excitation wavelength, 543nm; emission, 560nm long-pass filter) into orange-red cylindrical intravacuolar structures. ConA (excitation wavelength, 488nm; emission, 505nm long-pass filter) gives green fluorescence by binding to the cell wall glucose mannose and polysaccharides structures. The biofilms were transferred to fresh 35mm petri dish containing 2 ml PBS with 2µl of FUN-1 and 10µl of ConA working solution and incubated at 37°C for 35-45 min. After incubation the solution was removed and biofilm was observed with a Zeiss LSM510 confocal scanning laser microscope (Carl Zeiss, Inc., Germany).

Quantification of biofilm

XTT reduction assay

Biofilms were quantified by dry weight measurement and the XTT reduction assay as described earlier (6). The mitochondrial dehydrogenase enzyme reduces XTT salts (Sigma Chemical Co., St. Louis, MO) into a water-soluble formazan product giving orange color that is measured spectrophotometrically at 492nm. SE discs with mature 48h old *Candida* biofilms were transferred to a new 12-well tissue culture plate containing 4 ml of PBS per well (22). For the XTT assay, 50µl of XTT salt solution (1mg/ml in PBS) and 4µl of menadione solution (1mM in acetone; Sigma Chemical Co.) were added to each well and mixed gently. For 3 strains of *C. parapsilosis,* 50µl of a 5mg/ml XTT solution and 12µl of menadione were used due to low metabolic activity of these strains. The blanks contained sterile SE discs without any biofilm. Plates were then covered by aluminum foil and incubated at 37°C for 5h on the rocker table. After 5h, medium was removed from the wells of the 12 well plate and was centrifuged at 3000 rpm in order to remove fungal cells debris. XTT-formazan in the supernatant was measured at 492nm by using a spectrophotometer (Genesys 5; Spectronic Instruments, Rochester, N.Y).

Dry weight measurement

Dry weight measurement (DW) quantifies the total biofilm mass, which includes all the fungal cells and the extra cellular matrix. Biofilms were scraped off the surface of the discs by using a cell scraper (Becton Dickinson, USA). Discs and scrapers were rinsed with

PBS to remove left over biofilm. The material collected was filtered using a 0.45µm size Millipore filter, then air dried at 37°C for 48h, and weighed

Antifungal susceptibility of Candida biofilms

For the antifungal susceptibility of *Candida* biofilms, a 96 well plate method was used (23). 5mm discs of SE were cut using a cork borer and autoclaved after washing with soap and water according to manufacturer's instructions. SE discs were pretreated with FBS by incubating 8 discs per well in 12 well plate at 37°C for 24h, as described previously (23). Standard inoculum of 10^7cells/ml was made in PBS using a haematocytometer. The pretreated discs were transferred into the 12 well plate-containing 10^7cell/ml for 90min at 37°C for adhesion. After adhesion phase discs were carefully taken out using a sterile needle and were transferred into 96 well plates containing 200 µl of YNB per well and were incubated in a linear motion for 24h for maturation phase.

Caspofungin and fluconazole were obtained from Merck (Rahway, N.J, USA) and Pfizer Pharmaceuticals Group (New York, NY, USA), respectively. A stock solution of caspofungin was made in DMSO, while fluconazole is water-soluble. Serial dilutions ranging from (32-0.06µg/ml) were made in a 96 well plate in YNB. The 5mm discs with mature biofilms were transferred into the 96 well plate containing serial dilutions of drug by using sterile needles. Column 11 is the growth control having only mature biofilm and column 12 is the blank having no biofilm. A 200µl aliquot of working solution-containing 4ml of PBS with 50µl of XTT (1mg/ml) and 4µl of menadione was transferred into the 96 well plates. After 24 hours of treatment of the biofilms by the drugs, the 5mm SE discs were then transferred to a 96 well plate containing a working solution of XTT and menadione. The 96 well plates was covered with aluminum foil and incubated at 37°C on a rocker for 90 min. After 90min the plates were spun at 3000 rpm for 5 min. 100µl of the medium in the wells was taken carefully using a multi-channel pipette (Thermoscientific, USA) and transferred into a new sterile 96 well plate. The absorbance was taken calculated spectrophotometrically at 490nm by placing the plate in micro-titer plate reader (Bio-Rad, Japan). The reading by XTT–based assay was taken by comparing the percentage growth of the wells having no drug compared to the growth. The concentration that reduced the metabolic activity by ≥50% as compared with the growth control was taken as the minimum inhibitory concentration (MIC).

Statistical analysis

All the experiments were repeated three times in alternate days. The statistical analysis was done by a two-tailed Student's t-test using software SPSS version 22 to analyze the results. The *P* value greater than 0.05 was considered as significant.

Results

Biofilm Formation

Fluorescent Microscopy

Candida albicans

Figure 1a indicated the image of biofilm from a biofilm positive *Candida albican* SC-5314 (control strain). All the strains of *C. albicans* included in the study produced biofilms. The cells were seen as a mat of cells and matrix over the silicone elastomer discs (Fig. 1b). Extent of the biofilm formation also varied within the same species. Biofilms formed by the strains M-1993 and M-2524 were very dense. Strains M-619 and MRL-648 did not form uniform biofilms as these biofilms came off easily during calcofluor staining. Extra cellular matrix, which can be seen as haze, and a network of hyphae can be seen in the images.

Candida tropicalis

Biofilms formed by *C. tropicalis* had very heterogeneous structure. Morphologically, most of hyphae and pseudohyphae were covered by thick extracellular polymeric matrix. Strain A-2029 formed thick biofilm which was isolated from a vaginal specimen. It adhered tightly to the pretreated silicone elastomer discs during calcofluor staining. Strains of *C. tropicalis* produced a large amount of extra cellular matrix (Fig. 1b).

Table 1. Base line data of the *Candida* isolates.

No.	Lab. No.	Species	Source
1	A-985	*C. glabrata*	Urine
2	A-2700	*C. tropicalis*	Urine
3	MRL-648	*C. albicans*	Unknown
4	MRL-759	*C. tropicalis*	Unknown
5	M-2524	*C. albicans*	Vagina
6	A-2029	*C. tropicalis*	Vagina
7	M-1993	*C. albicans*	Vagina
8	M-159	*C. glabrata*	Urine
9	A-2407	*C. glabrata*	Urine
10	A-1765	*C. glabrata*	Urine
11	M-2954	*C. glabrata*	Vagina
12	MRL-2006	*C. parapsilosis*	Unknown
13	A-1554	*C. glabrata*	Urine
14	M-3212	*C. tropicalis*	Vagina
15	M-619	*C. albicans*	Vagina
16	M-3166	*C. tropicalis*	Urine
17	A-1446	*C. parapsilosis*	Urine
18	A-2512	*C. glabrata*	Stool
19	M-4369	*C. albicans*	Sputum
20	A-1439	*C. glabrata*	Urine
21	A-3189	*C. tropicalis*	Urine
22	M-1470	C. *kruseii*	Urine
23	A-2686	*C. glabrata*	Vagina
24	MRL-17351	*C. glabrata*	Unknown
25	M-530	*C. parapsilosis*	Vagina

Candida glabrata

C. glabrata also demonstrated the ability to produce biofilm, but the biofilms formed by *C. glabrata* were layers of blastopores. There was no hyphal layer in biofilms of *C. glabrata* because of the inability to produce hyphae (Fig. 1c). It usually represented overlapping layers of blastospores adhering to the FBS treated silicone elastomer sheet. Some strains of *C. glabrata* form biofilms, which are seen as scattering adhering blastospores over the surface of the pretreated SE disc. Among 8 strains of the *C. glabrata,* only 3 produced biofilms. The biofilm could be seen as communities of blastospores adhering in a scattered pattern over the surface of SE discs, while the rest of strains did not form biofilms.

Candida parapsilosis

Strains of *C. parapsilosis* included in this study produced biofilms. Structurally, a thick compact arrangement of the blastospores covered with haze; hyphal forms were visible scarcely. The extra cellular matrix covered the whole biofilm as shown in figure 1d. The biofilm was closely packed and a dense structure was observed by the fluorescence microscopy.

Confocal Scanning Laser Microscopy (CSLM)

Control Strain (SC-5314)

The CSLM images of the SC-5314 strain showed that the biofilm had a heterogeneous architecture (2a). A very fine network of hyphae was observed under the

CSLM. The overall thickness biofilm was 55 µm with thin areas of metabolically active cells intermingled with extracellular polysaccharide material, as indicated by deep red color on FUN-1 staining. Individual blastospores were not seen and the whole biofilm was made up of intertwining hyphae. Extracellular matrix was much more than metabolically active parts which could also be observed as haze in fluorescence microscopy.

Candida albicans

The biofilm of *C. albicans* has a heterogeneous structure, representing both blastospores as well as hyphal forms (Fig. 2b). The blastospores form is much more prevalent than the filamentous form and a lot of haze can be observed which is due to the extracellular matrix sugars. The thickness of biofilm was 23 µm which is much less than the SC5314 control strain.

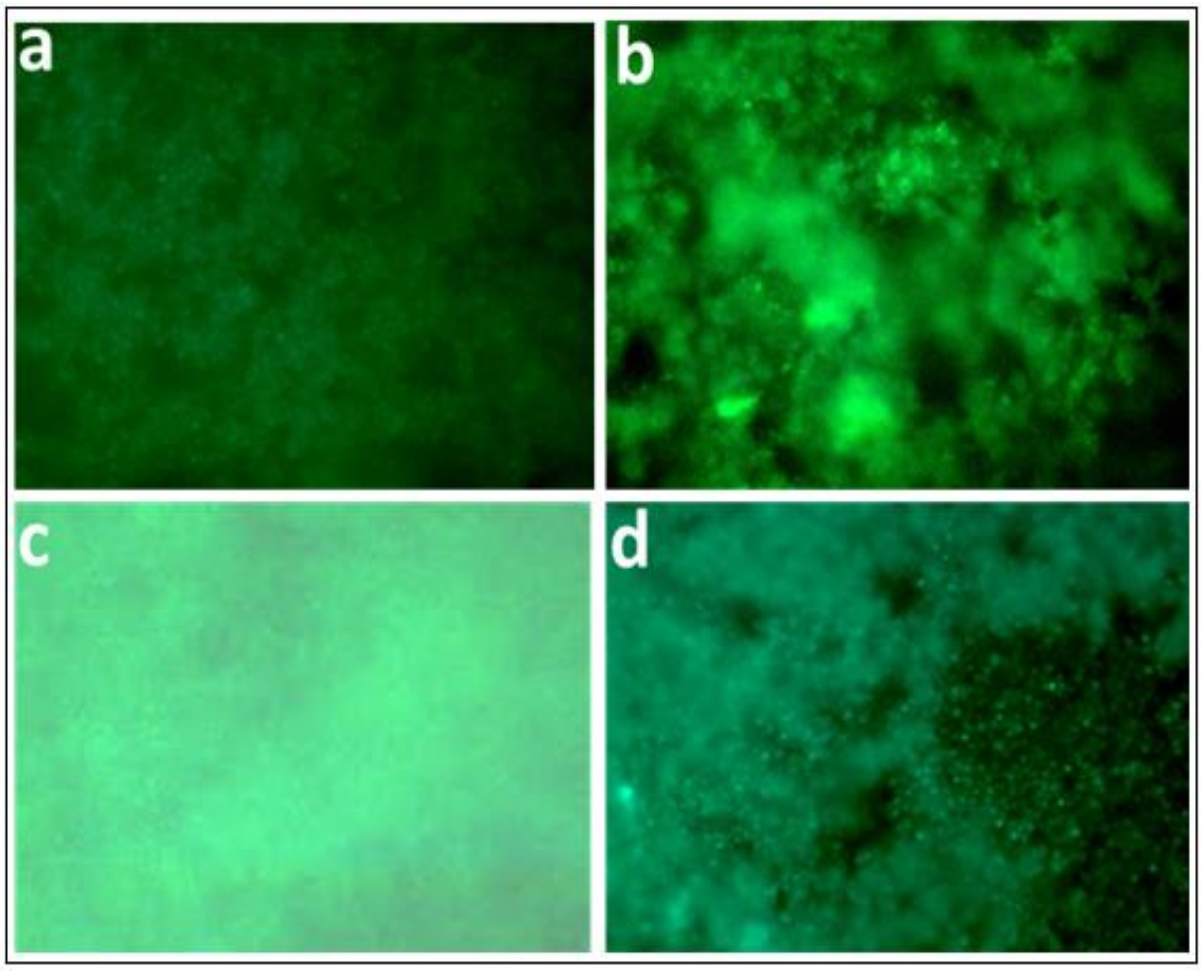

Fig. 1. Flourescence microscopy images of different *Candida* sp. (a) *C. albicans*, (b) *C. tropicalis*, (c) *C. glabrata*, (d) *C. parapsilosis*.

Candida tropicalis

C. tropicalis strain A-2029 produced a very dense biofilm. Both yeast cells and hyphae contributed in the construction of biofilm, as shown in figure 2c. Blastospores are abundant than the hyphal forms. The mean thickness of this biofilm was 41 µm. The main portion of biofilm was comprised of metabolically active cells, as indicated by red fluorescence in the image.

Candida glabrata

The image of *C. glabrata* was obtained by stacking together the images of different layers of the depths of the biofilms (Fig. 2d). The mean thickness of biofilm was 35 µm. No filamentous forms were present in this biofilm. C. glabrate biofilm was largely made up of polysaccharides, observed as dense green fluorescence in the image as compared to red coloration.

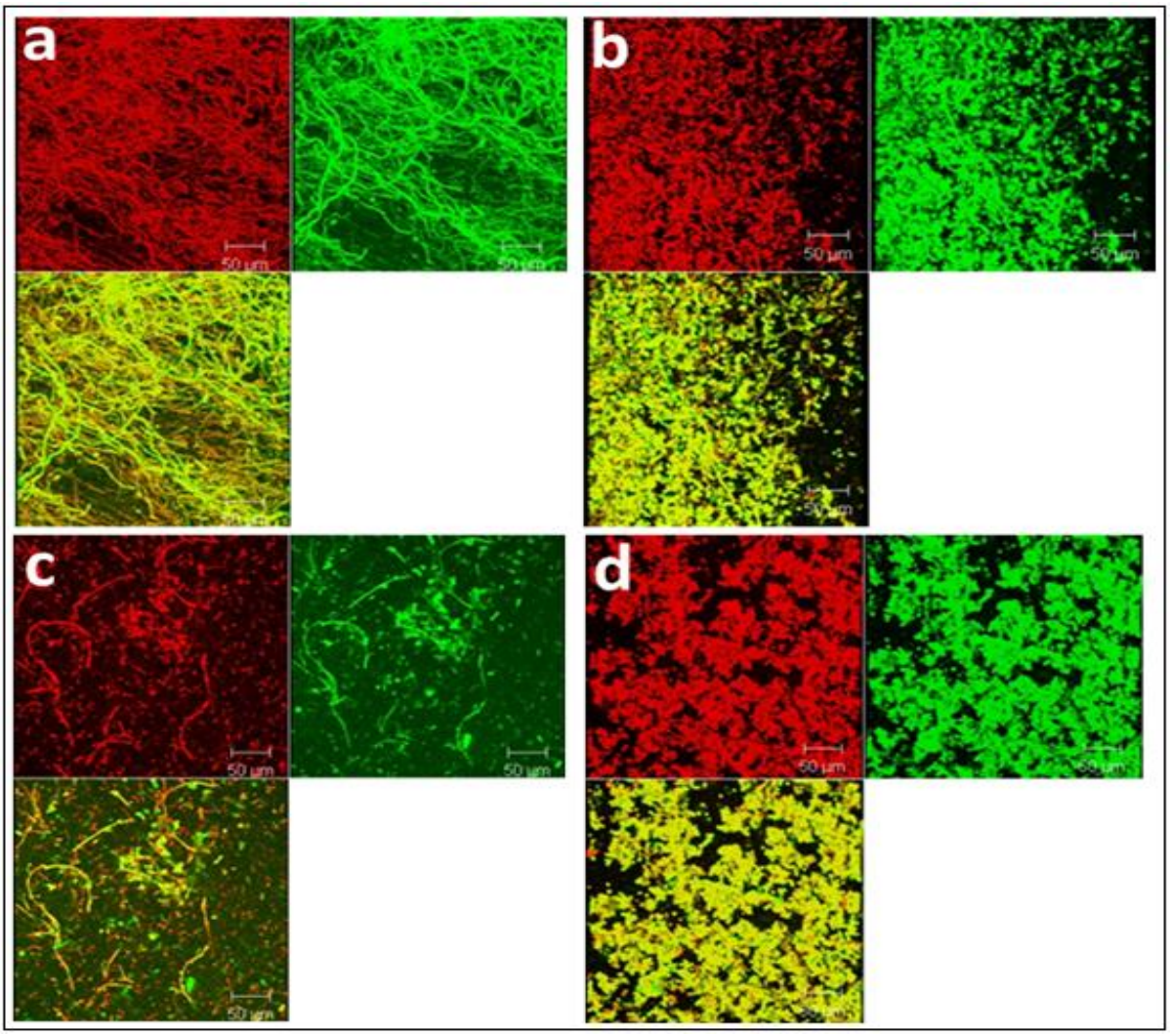

Fig. 2. Showing confocal scanning laser microscopy images of different biofilm of *Candida* species (a) Control SC 5314 *C. albicans* (b) *C. albicans* (c) *C. tropicalis* (d) *C. parapsilosis* (e) *C. glabrata*.

The large spaces among cells could be seen under high magnification, which resulted in non-uniform structure of biofilm.

Candida parapsilosis

C. parapsilosis biofilm was mainly made up of compactly packed blastopores as compared to hyphal forms (Fig. 2e). The green and red color fluorescence was almost equal in density, indicating that the biofilm was equally comprised of cytoplasm and the cell wall polysaccharides. The overall thickness of the biofilm was 55 µm.

Quantification of biofilm

Biofilms from different *Candida* sp. was quantified by XTT reduction assay and measurement of dry weight.

Candida albicans

The highest metabolic activity was recorded for the strain M-4369 isolated from sputum, as compared to other strains. *Candida albicans* strain MRL-648 from the Mycology Research Laboratory, University Hospital Medical Center, Cleveland, and showed low activity as compared to the other strains as well as the control strain SC-5314. Maximum dry weight was calculated for the biofilm formed by strain M-2524 (6mg) as compared to other strains (Fig. 3). The total biomass produced by the control strain was 3.7 mg.

Candida tropicalis

The highest biomass and metabolic activity was calculated in case of strain 2029, isolated from vaginal swab (Fig. 3). The strains A-2029 and A-3212 isolated from HVS samples gave a high absorbance as compared with the strains isolated from urine. Strain

M-3166 from MICU patient demonstrated higher metabolic activity as compared with the other strains isolated from the urine samples.

Candida glabrata

The XTT assay results of *C. glabrata* strains showed low absorbance as compared to the control strain. Only three strains A-985, A-2954 and A-1765, formed biofilms. Although *C. glabrata* grew well in the YNB, most of the strains were unable to form the biofilm. Low biomass was calculated in case of the strains of *C. glabrata* (less than 2mg) as they were unable to adhere to the FBS treated silicone elastomer discs (Fig. 3).

Candida parapsilosis

Though the strains of *C. parapsilosis* form good biofilms as indicated by FM images, however, they were metabolically less active and therefore their assay was performed with a higher concentration of XTT salts and menadione, finally gave high XTT reduction assay values. The biomass was as greater than 2.5mg but still less than that of control strain (Fig. 3).

Antifungal susceptibility of Candida biofilms

The antifungal susceptibility was checked for two antifungals, fluconazole (0.5µg/ml-256µg/ml) and caspofungin (0.015µg/ml-32µg/ml). The fluconazole MIC biofilms of all *Candida* sp. was greater than 256µg/ml, indicating that all *Candida* biofilms were resistant to fluconazole. In contrast, caspofungin demonstrated low MIC values, showing activity against the biofilms. However, the strains of *Candida* that were resistant to caspofungin in planktonic form were also resistant in biofilms.

Statistical analysis

T-test was applied on the XTT reduction assay and dry weight results of *Candida* sp. It was found that the biofilms formed by the *Candida albicans*, *C. tropicalis* and *C. parapsilosis* were not significantly different as the *P* value was lesser than 0.05 while these biofilms were significantly different from *C. glabrata* as the *P* value was >0.05.

Discussion

Biofilms are the mode of living of microbes that increases their survival by becoming more adaptive to the environment. In nature, microbes prefer to live as biofilms, whereas somewhat planktonic or free floating forms are very uncommon (24). *Candida* species have an ability to cause infections by formation of biofilms. These biofilms are made up of microcolonies of blastospores and hyphae that are enclosed in a matrix (4). Earlier studies showed that *Candida* biofilms were formed in 3 distinct phases, earlier, intermediate and mature. The earlier phase is accompanied by adhesion of the cells over the surface of the substrate. The intermediate phase involves the proliferation of the cells, and the final phase is the maturation which proceeds by development of hyphae and pseudohyphae (6). This study was conducted to compare the biofilms formed by the 4 common *Candida* sp., *C. albicans*, *C. tropicalis*, *C. glabrata* and *C. parapsilosis*. All conditions were set to favour the biofilm formation.

Interspecies and intraspecies variation in the structure of biofilms has been observed in previous reports using fluorescence and confocal scanning laser microscopy (CSLM). The chief advantage of this technique is that the whole architecture of the biofilm is observed without affecting the viability of the yeast cells (25,26). The results indicated that biofilms from *Candida albicans* were covered by extra cellular matrix, forming a capsule around the blastospores, hyphae and pseudohyphae. Seneviratne *et al.* (27) and Chandra *et al.* (6) reported that most of *Candida biofilms* comprised both hyphae and blastospores. In case of *C. tropicalis* the biofilms carried yeast forms with very few thin structure hyphae surrounded by extra cellular matrix. Kuhn *et al.,* (21) reported that the biofilm of *C. tropicalis* is made up of thin hyphae during early stages but later on develops blastospores after maturation. Most of the biofilms from *C. tropicalis* are made up of microcolonies of blastospores and covered with a thick extra polymeric layer (28). The FM images of the *C. glabrata* showed that these biofilms are made up of blastospores with no hyphal switches. Silva *et al.* (29) and Kucharikova

et al. (14) reported multilayered biofilms of *C. glabrata* that mainly comprised of clusters of blastospores scatterly adhered to the SE discs. *C. parapsilosis* biofilm usually appear as aggregates of blastoconidia on a basal layer (12). *C. parapsilosis* formed dense biofilms comprised of blastospores in a compact cover of extracellular matrix. A Silva *et al.* (29) reported dense biofilm with yeast and pseudohyphae from *C. parapsilosis*.

The biofilms were characterized by using XTT reduction assay and dry weight determination. According to Seneviratne *et al.* (27), single quantification method is not enough for characterizing of biofilms as XTT assay has some pitfalls. XTT is inexpensive reproducible quantitative method that determines the viability of the cells by their ability to reduce XTT by forming formazan product (30). Strains of *C. albicans* and *C. tropicalis* gave high XTT assay values as compared to *C. glabrata*, that revealed the formers are the good biofilm producers. *C. parapsilosis* biofilm with low metabolic activity, therefore, high concentration of XTT salts were used (12). *C. albicans* produced highest quantity of biomass as compared to *C. tropicalis* and *C. parapsilosis* as indicated by the dry weight analysis. *C. albicans* produce thick biofilm on SE discs as compared to the non-albicans, which has been confirmed from the previous reports (21,20,31).

Although the cell count and CFU was high in case of *C. glabrata* but the dry weight was low with respect to other *Candida* sp (data not shown). Usually, *C. albicans*, *C. parapsilosis* and *C. tropicalis* are good biofilm formers as compared other *Candida* species (29,31).

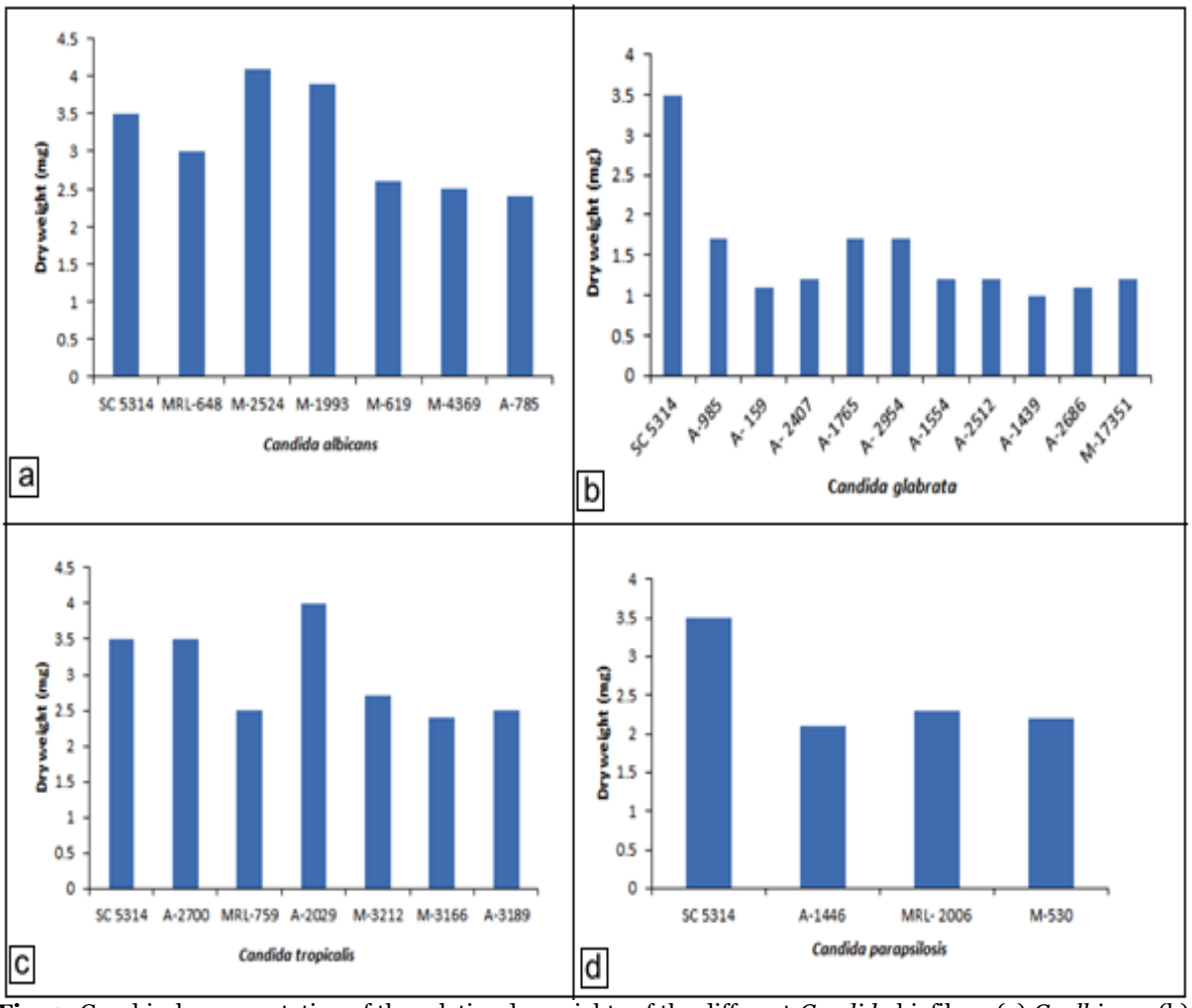

Fig. 3. Graphical representation of the relative dry weights of the different *Candida* biofilms. (a) *C. albicans* (b) *C. glabrata* (c) *C. tropicalis* (d) *C. parapsilosis.*

The antifungal susceptibilty testing of the biofilms was done by the 96 well plate method, which has an advantage in that it can be used to check the antifungal susceptibility on live catheter biofilms (23). Antifungal sensitivity was check against Fluconazole and caspofungin . All of the *Candida* biofilms formed by strains tested proved to be resistant to fluconazole (MIC_{50} > 256µg/ml). By contrast, caspofungin proved to be effectve against all the biofilms of *C. albicans*. The antifungal suceptibility testing done on *Candida* biofilms by Fiori *et al.*, (33) and Ramage *et al.* (34) a also reported the failure of azoles against the *Candida* biofilms. Kuhn *et al.*, (21) also found that caspofungin was effective aginst biofilms of *Candida* species. The strains which were intermediately caspofungin-resistant in planktonic form were resistant to it in the biofilm form. *C. parapsilosis* strain 2006, which was highly resistant to caspofungin in planktonic form (MIC > 64 µg/ml) was also resistant to it in the biofilm form. Furthermore, all the strains of *C. glabrata* tested had high MIC values against caspofungin that confirms the study of Seneviratne *et al.,* (35) who reported that the efficacy of caspofungin was dependent upon the strains and the biofilms produced by the strains resistant to caspofungin have higher MIC values.

Conclusions

From our results we have concluded that *Candida* species have the ability to form biofilms of variable morphology and architecture probably due to the physiological differences. CSLM is a new technique that helps to provide an insight of the biofilm structures. Dry weight and XTT assays are simple reproducible methods to analyse the biofilms. The XTT assay has some limitations, but together with dry weight, it is a useful technique for characterization of biofilms. Fluconazole proved to be uneffective against the *Candida* biofilms tested. This data indicates that caspofungin is the drug of choice for treatment of the *Candida* infections due to biofilm fomation.

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