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## Measuring susceptibility of *Candida albicans* biofilms towards antifungal agents

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### ABSTRACT

Biofilm drug resistance may explain the persistence of many infections in the face of appropriate antimicrobial therapy. Sixty four isolates of planktonic *Candida albicans* revealed high incidence of resistance to azoles than polyenes antifungal agents. A semiquantitative assay was used to measure the metabolic activity of *C. albicans* biofilms treated with the antifungal agents and then determine the sessile minimum inhibitory concentration of the antifungal agents which caused 50 % inhibition of the formed biofilms (SMIC<sub>50</sub>). There was dramatically increase in antifungal concentrations required to kill *C. albicans* isolates in the sessile forms compared to planktonic forms. Kinetics of biofilm formation showed that the biofilms were highly metabolically active after the first 12 hours. A severe drop in the finally formed biofilms was obtained by adding nystatin at a concentration equal to sub MIC of planktonic cells. On exposure of the biofilms during their formation to nystatin at a concentration equal to sub SMIC<sub>50</sub> of formed biofilm cells after 3 and 6 hours, there was a severe drop in the finally formed biofilms. Adding nystatin after 12 and 24 hours, the formed biofilms became more metabolically active. Scanning electron microscope (SEM) revealed that the fully mature biofilm was produced after incubation for up to 48 hour. Growing the biofilm from the beginning with nystatin at SMIC<sub>50</sub> resulted in a large reduction in cell numbers and in the thickness of the biofilm compared to the normal biofilm.

**Keywords:** *Candida albicans*; biofilms; antifungal susceptibility.

### INTRODUCTION

Biofilms are composed of populations or communities of microorganisms adhering to environmental surfaces. These microorganisms are usually encased in an extracellular polysaccharide that they themselves synthesize [1]. Biofilms can serve as a cause for disease and are often associated with high-level antimicrobial resistance of the associated organisms [2,3]. A wide range of biomaterials used in clinical practice support colonization and subsequent biofilm formation by *Candida* spp. [4].

*Candida* is the fourth most common cause of blood stream infection in hospitalized patients. Up to 40% of patients with candida isolated from intravenous catheters have underlying fungemia, and the mortality rate of patients with catheter-related candidemia approaches 40% [5, 6]. *Candida albicans* is the most commonly isolated fungal species and it is the major fungal pathogen of humans [7,8].

Biofilms of *C. albicans* are capable of holding other micro-organisms and more likely to be heterogeneous with other bacteria and fungi in the environment and on medical devices [6,9]. Biofilm cells are significantly less

susceptible to antimicrobial agents. As a result, drug therapy for an implant infection may be futile, and often, the only solution is mechanical removal of the implant [10,11].

In this time of widespread increased antimicrobial resistance and increased use of indwelling devices, the objective of this work was to study the effect of different antifungal drugs on the preformed *C. albicans* biofilms.

## MATERIALS AND METHODS

### Microbial strains

Ninety and nine urine samples were collected from patients suffering from urinary tract infections. Also, 100 vaginal discharge samples of female patients suffering from itching and vaginitis were collected. All these specimens were obtained from Beni-Suef Hospital Health Insurance, Beni-Suef university Hospital and Beni-Suef Republic Hospital, Beni-Suef city, Egypt. The culture characters and the color of the developed colonies were used to identify *C. albicans* as described by [12].

### Susceptibility determination of *C. albicans* isolates to different antifungal agents

Susceptibility of *C. albicans* isolates was determined by the agar dilution method on Roswellv Parkv Memorial Institute (RPMI) 1640 medium (Sigma Chemical Co., USA) towards 11 antifungal agents as recommended by [13]. The antifungal agents were : a) polyenes; amphotericin B (AMP) , nystatin (NYS), b) azoles; fluconazole (FLZ), ketoconazole (KTZ), itraconazole (ITZ), miconazole (MIZ), econazole (ECZ), tioconazole (TCZ), clotrimazole (CTZ) and sertaconazole (SRZ) and c) allylamines: terbinafine (TRB). The results of growth were recorded knowing that the minimum inhibitory concentration (MIC) is defined as the lowest concentration of the antifungal agent giving no visible growth or causing almost complete inhibition of growth.

### Induction of candida biofilm formation

As previously described by [10], eight *C. albicans* isolates, 4 urine isolates and 4 vaginal isolates with different resistance patterns, were selected. Isolates CA27 and CA137, represented resistant isolates, while isolates CA47, CA66 and CA189 were intermediately resistant isolates and CA33, CA141 and CA150 isolates represented sensitive isolates. *C. albicans* biofilms were formed on commercially available presterilized, polystyrene, flat-bottomed, 96-well microtiter plates (Corning Incorporated, Corning, NY). Biofilms were formed by pipetting standardized cell suspensions (100  $\mu$ l of  $1 \times 10^6$  cells/ml) into selected wells of a microtiter plate. The 12th column of wells on the plate should remain empty, negative control. The microtiter plates were covered with their lids, sealed with parafilm, and incubated for 48 hr at 37°C. After biofilm formation, the RPMI medium was aspirated. Planktonic and nonadherent cells were removed by thoroughly washing the biofilms with sterile phosphate buffer saline (PBS).

### Challenging of preformed candida biofilms with antifungal agents

Final working concentrations of each antifungal agent in the range of 20-2000 X of the pre-determined planktonic MICs were prepared in RPMI medium. Two hundreds  $\mu$ l of the high working concentration of the used antifungal was added to the first well of the microtiter plate containing fungal biofilms. One hundred  $\mu$ l of RPMI per well was added to wells 2–10. Two hundreds  $\mu$ l of RPMI was placed in well 11 as a positive control. One hundred  $\mu$ l of antifungal agent in the first well was then removed and added to the RPMI of the second well. The last step was repeated up to the tenth wells, the final 100  $\mu$ l volume was discarded. The plates were then covered with their lids, sealed with parafilm, and incubated for 48 hr at 37°C. After antifungal challenge, biofilms were processed and washed with sterile PBS [14].

### Use of a colorimetric metabolic assay as a semiquantitative method to assess fungal cells viability after treatment with antifungal agents

As described by [15] one hundred  $\mu$ l of the XTT/menadione solution, (2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide, (Sigma Co.,USA) was added to each pre-washed biofilm and to the negative control wells. The plates were then covered with aluminum foil and incubated in the dark for 3 hrs at 37°C. After incubation, 75  $\mu$ l of XTT dye from each well was removed and transferred to the wells of a new microtiter plate (without biofilms). These plates were then read in a microtiter plate reader at 490 nanometer (nm). If XTT was effectively reduced by live yeast cells, the originally clear solution was transformed into an orange color.

### Kinetics of biofilm growth

*C. albicans* biofilms formation were initiated in microtiter plates as described before for 3 different *C. albicans* isolates; a resistant (CA27), an intermediate (CA66) and a sensitive (CA141) isolate. Biofilms were formed over a series of time intervals 3, 6, 12, 24 and 48 hr. At each time interval, biofilm formation was measured with the XTT assay. For each time interval, 3 biofilm replicates were formed. Repeating the same experiment for the three selected

isolates (CA27, CA66 and CA141) but in presence of NYS with RPMI media at a concentration equal to sub MIC of the planktonic cells (2.3 µg/ml, 4.6 µg/ml and 0.6 µg/ml, respectively)

To investigate the disturbance of biofilm formation due to the addition of NYS from the beginning the following experiment was carried out. Formation of *C. albicans* biofilm was initiated as described before for isolate CA66. Biofilms were formed over a series of time intervals (3, 6, 12, 24, 48 hr). At each time interval, biofilm formation was measured with the XTT assay. Repeating the same experiment for the same isolate but by applying different conditions by growing the biofilm normally for 3, 6, 12 and 24 hr then replace the RPMI media with RPMI media containing sub SMIC<sub>50</sub> of the biofilm cells (1156 µg/ml) at each time interval.

#### **Scanning electron microscopy of *C. albicans* biofilm before and after antifungal challenging**

Biofilms of *C. albicans* were formed on polycarbonate membrane filters then, four treatments were used. The biofilms were mounted onto standard EM stubs coated with double-sided, carbon black sticky tabs. The samples were air dried for 24 hr and coated with gold/palladium by using Polaron cool-sputter coater then examined by SEM using an accelerating voltage of 10 kV.

### **RESULTS AND DISCUSSION**

A total of 64 isolate of *C. albicans*, 16 and 48 from urine and vaginal samples respectively, were recovered from 199 different clinical specimens from Beni-Suef city, Egypt.

#### **Susceptibility and MIC determination for antifungal agents of planktonic and biofilm-associated *Candida albicans*.**

One of the most important observations was moderate resistance to CLZ, AMP, NYS and ECZ (1.6, 2.3, 3.5 and 3.7 µg /ml, respectively). On the other hand, a high incidence of resistance to FLZ, ITZ and KTZ was observed (58.3, 14.9 % and 14.1 µg/ml, respectively) (Table 1).

In vitro activity of clinically used FLZ, AMP, NYS, SRZ, MIZ, ECZ, KTZ and TRB against 8 pre formed biofilms using RPMI was assessed using the XTT-reduction assay. AMP and NYS showed activity against planktonic isolates in concentrations ranged between 0.5 and 9.25 µg/ml, while, their SMIC<sub>50</sub> were 31.25 to 4625 µg/ml. The activities of the other antifungal agents were ranged between 0.125 – 64 µg/ml, while their activities against biofilms (SMIC<sub>50</sub>) ranged between 31.25 – > 4000 µg/ml (Table 2).

#### **Kinetics of biofilm growth and formation on microtiter plates**

The biofilms were highly metabolically active after the first 12 hr, but as the biofilm matured and the complexity increased (24 to 48 hr), the metabolic activity reached a plateau, but remained high probably reflecting the increased number of cells that constituted the mature biofilm. The kinetics of adherence and subsequent biofilm formation by the three selected isolates but in presence of NYS at a concentration equal to sub MIC of the planktonic cells showed a marked decrease in the complexity and the cellular density of the formed biofilms for each isolate compared with the normal biofilm (Fig.1). Disturbing the biofilm formation of isolate CA66 after 3, 6, 12 and 24 hr using NYS at a concentration equal to sub SMIC<sub>50</sub> of biofilm cells resulted in an obvious decrease in metabolic activity of the finally formed biofilm compared with the normal biofilm which was seen only in case of adding NYS after 3 and 6 hr from starting normal biofilm formation (Fig.2). While in case of adding NYS after 12 hr, it showed transient decrease in metabolic activity after 24 hr and final increase in metabolic activity after 48 hr compared with that in absence of NYS (Fig.2).

#### **Scanning electron microscopy of *Candida albicans* biofilm before and after antifungal challenging**

There was an initial attachment of yeast cells was followed, after 3–6 hr (Fig.3a). Fully mature biofilms, produced after incubation for up to 48 hr (Fig.3d). The effect of NYS in its sub SMIC<sub>50</sub> of biofilm cells was also investigated by SEM after incubation for additional 24 hr. There was a wrinkled, ruptured, and ballooning effect of the drug on yeast cells (Fig.3e). Growing the biofilm from the beginning in NYS at sub SMIC<sub>50</sub> of biofilm cells was also scanned by electron microscope after 24 and 48 hr incubation. It resulted in a large reduction in cell numbers and in the thickness of the biofilm compared to the normal biofilm and several of the cells appeared swollen (Fig.3 f).

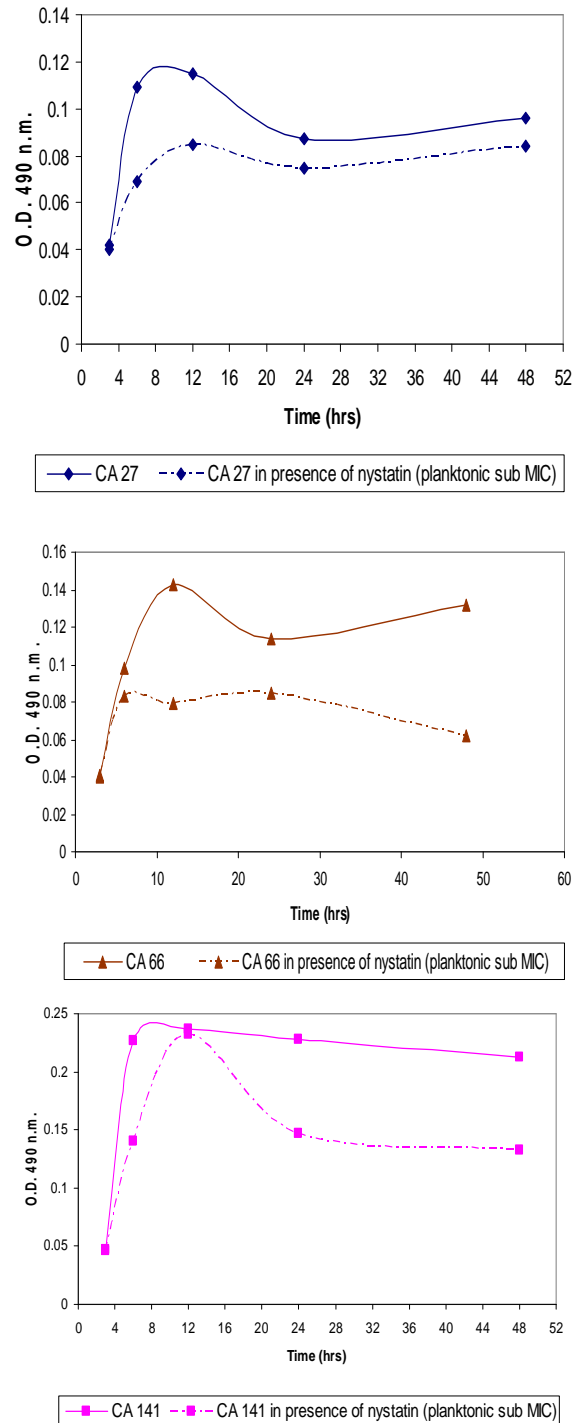
Biofilm formation plays an important role in outbreaks of *C. albicans* related infections [8]. Importantly, once formed, mature biofilms are very difficult to completely eradicate, at least in part because cells within a fully developed biofilm often exhibit drug resistance [16]. Consequently, contaminated devices must often be removed from the patient to prevent recurrent infections [17].

In the present study, the RPMI 1640 medium was selected since it has been evaluated extensively for in vitro susceptibility testing of yeasts and has been shown to provide reproducible results [18]. The obtained results showed that the MICs of nystatin were within the range of 0.125 – 9.25 µg/ml. These values were lower compared to those reported by [19] who noticed that the MICs of most *C. albicans* isolates to nystatin as determined by the same method were in the range of 4 – 16 µg/ml. Amphotericin B MICs were within the range of 0.5 – > 16 µg/ml and these results were higher related to that published by [20]. The mean MIC of clotrimazole was 1.6 µg/ml, almost the same result was observed with econazole and tioconazole with mean MIC not exceeding 3.7 µg/ml and 5.9 µg/ml, respectively. These results were in line with those reported by [21]. Also, the MICs ranges of ketoconazole and itraconazole were ranged between 0.25 – > 16 µg/ml like those obtained by [22].

In this study, we used the XTT assay to measure the metabolic activity of 48-hr *C. albicans* biofilms treated with the antifungal agents and then determine the sessile MIC of antifungal which cause 50 % inhibition of the formed biofilm (SMIC<sub>50</sub>). Amphotericin B showed SMIC<sub>50</sub> ranged between 31.25 – >1000 µg/ml while it was only between 0.5 – 64 µg/ml against the planktonic cells. Also, the biofilms showed a drastic resistance towards fluconazole SMIC<sub>50</sub> was often > 2000 µg/ml. Baillie and Douglas [23] observed that 20 times the MIC of commonly used antifungals such as amphotericin B, fluconazole, or flucytosine was required to cause a significant reduction in cell numbers. Bachman and coworkers [8] reported that for fluconazole, the absorbance readings of the XTT-reduction assay in biofilms at concentrations as high as 1,024 µg/ml were similar to those of the control biofilms (no drug). The activity (SMIC<sub>50</sub>) against biofilms by other antifungal agents like nystatin, ketoconazole, miconazole, econazole, tioconazole and sertaconazole, it ranged between 31.25 – > 4000 µg/ml. Our results corroborate previous observations indicating the increased resistance of adherent populations of *Candida* cells to clinically used antifungal agents [20, 24]. Despite the improved activity of some newer antifungal agents, complete eradication of sessile organisms within mature biofilms has not been demonstrated [1]. Fortunately, simple removal of a catheter in addition to antifungal therapy helps resolve the infection in many cases. During *C. albicans* infection, arachidonic acid (AA) is released from phospholipids of infected host cell membranes and used by *C. albicans* as the sole carbon source and for production of eicosanoids. It is suggested that the effectiveness of polyene and imidazole antifungals may depend upon the level of unsaturation and ergosterol in the membrane [25].

In an effort to determine if antifungal agents could affect the formation of mature biofilms, our results showed a marked decrease in the complexity and the cellular density of the formed biofilm for each isolate compared with the normal biofilm. On a clinical level, these results may point to approaches for preventive or prophylactic treatment. These findings were the same as suggested by [26], who stated that, local low (hence non-systemic) levels of antifungal agents may be sufficient to inhibit or disrupt biofilm formation. Adding nystatin after 3 and 6 hr during biofilm formation at a concentration equal to sub SMIC<sub>50</sub>, there was a severe drop in the finally formed biofilm. While adding nystatin after 12 and 24 hr gave an illogic results where the formed biofilm became more metabolically active compared to the normal biofilm. These findings were consistent with those reported by [27] who noted in their experiments that there was a paradoxical rise in metabolic activity of mature biofilms at the highest concentrations of caspofungin. An interesting possibility to explain these findings had been proposed by [26] that the majority of cells within the biofilm were not necessarily more resistant to killing than planktonic cells, but rather a few persisters survived and were actually preserved by antibiotic pressure. Our suggestion was that, these persisters may have more ability to grow within the biofilm than the normal cells.

Biofilm formation by *C. albicans* on polycarbonate membrane filter was monitored by SEM. *C. albicans* We observed large numbers of *C. albicans* microcolonies on the filter surface after only 3 hr of incubation. Upon prolonged incubation, *C. albicans* biofilms developed into a dense mass stacked in a palisadic manner devoid of intracellular spaces. A large reduction in cell numbers and in the thickness of the biofilm after growing of the biofilm from the beginning in nystatin at subinhibitory concentration of the planktonic cells and scan it by SEM was observed which confirm the previous findings by [23, 28], who showed that the growth of *C. albicans* in medium containing a sub-inhibitory concentration of antifungal agent induced a decrease of subsequent fungal adherence, corresponds to the early phase of the biofilm formation. There was a wrinkled, ruptured, and ballooning effect of the drug on yeast cells after applying nystatin in its inhibitory concentration on 24-hr biofilms.



**Fig.1: Comparison between growth kinetics of *C. albicans* biofilm in presence of nystatin at a concentration equal to sub MIC of planktonic cells and that in absence of nystatin (normal biofilm). A: kinetics of isolate CA27, B kinetics of isolate CA141 and C kinetics of isolate CA66.**

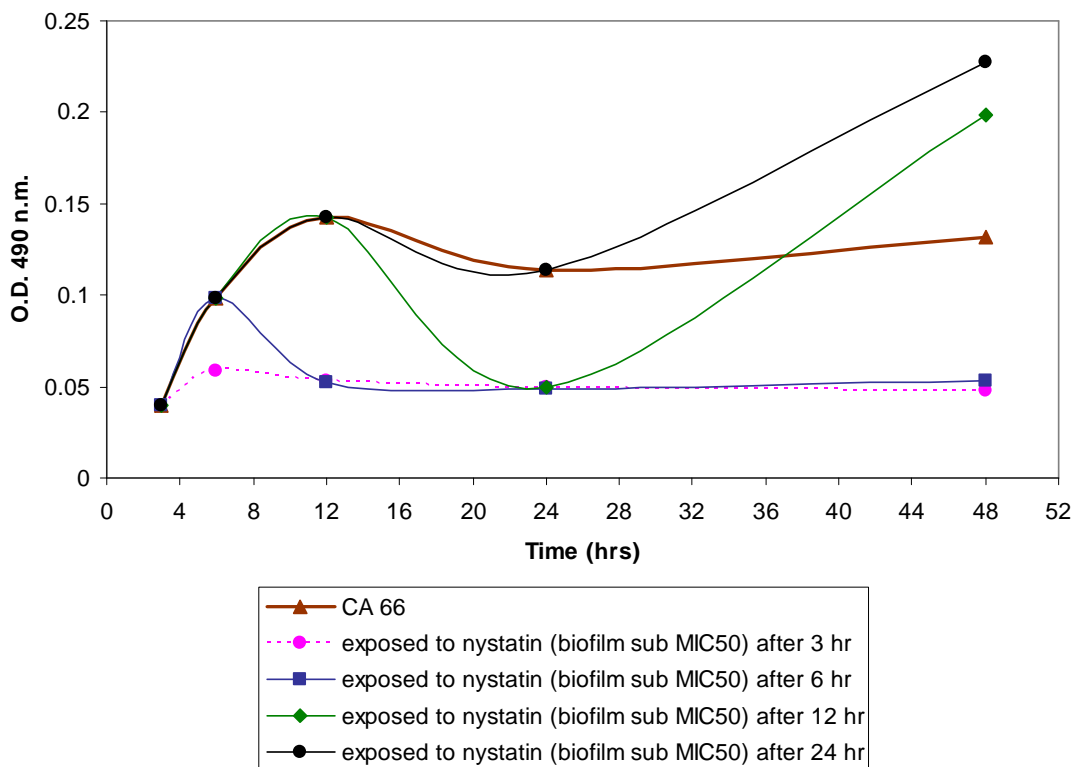


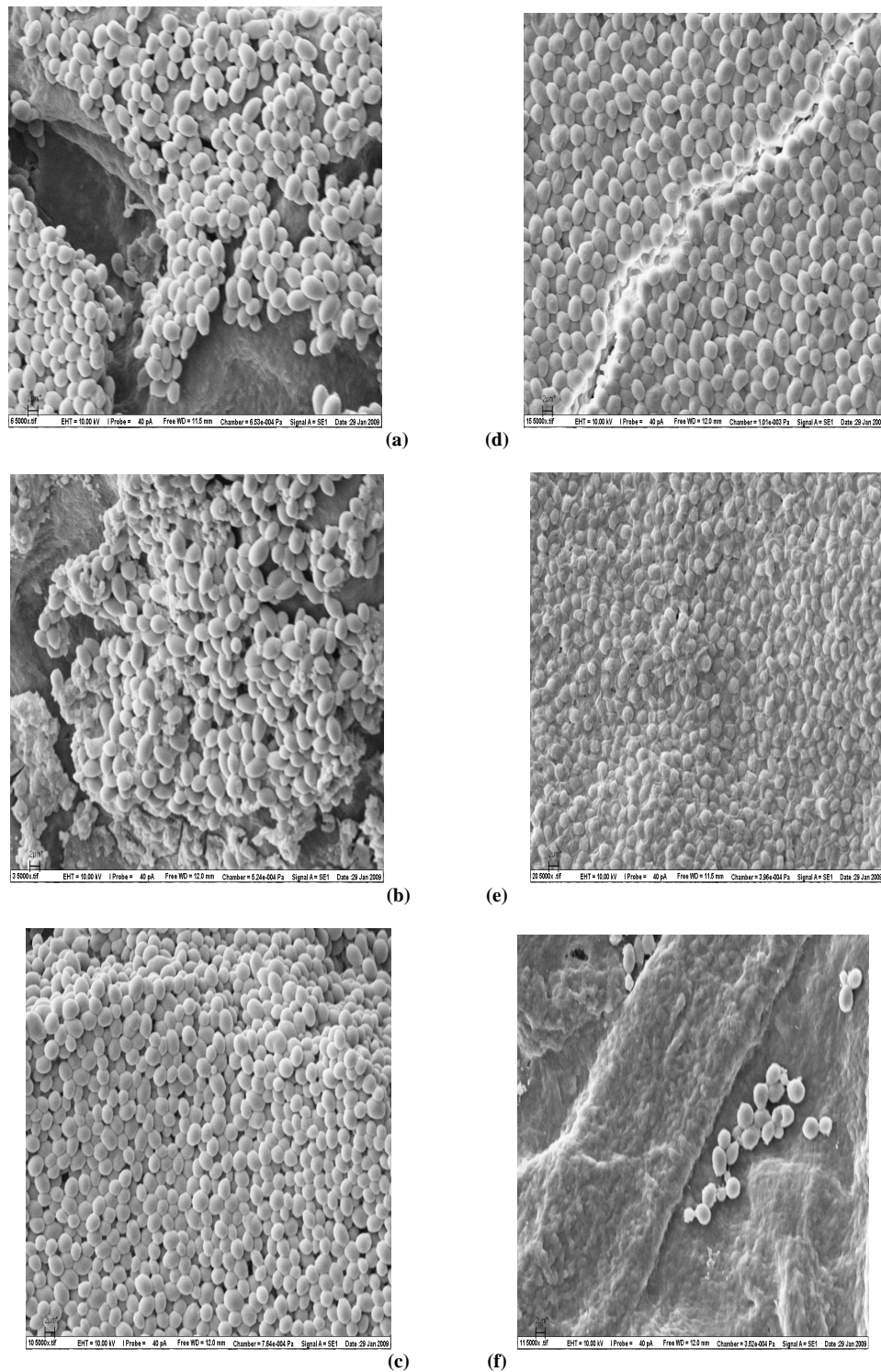
Fig.2: Effect of adding nystatin at concentration equal to biofilm sub SMIC<sub>50</sub> at different times after starting biofilm formation compared with normal biofilm.

Table 1: Minimum inhibitory concentrations (MICs) of the tested antifungal agents against planktonic *C. albicans*:

Antifungal Group	Antifungal agent	MIC Range (µg/ml)	Mean MIC (µg/ml)
Polyenes	Amphotericin B (AMP)	0.5 – > 16	2.3
	Nystatin (NYS)	0.125 – 9.25	3.5
Azoles	Ketoconazole (KTZ)	0.25 – > 16	14.1
	Itraconazole (ITZ)	0.25 – > 16	14.9
	Fluconazole (FLZ)	0.25 – > 64	58.3
	Miconazole (MIZ)	0.125 – 16	4.2
	Econazole (ECZ)	0.125 – 16	3.7
	Sertaconazole (SRZ)	0.5 – > 16	7.7
	Clotrimazole (CLZ)	0.25 – 8	1.6
	Tioconazole (TCZ)	0.06 – > 16	5.9
Allylamines	Terbinafine (TRB)	1 – > 64	10.4

Table 2: Minimum inhibitory concentrations of antifungal agents against planktonic and biofilms-associated *Candida albicans*:

Isolate no.	CA27		CA33		CA47		CA66		CA137		CA141		CA150		CA189	
	0.404		0.44		0.455		0.52		0.63		0.832		0.91		0.628	
OD <sub>490nm</sub>	PMIC*	SMIC <sub>50</sub> *	PMIC*	SMIC <sub>50</sub> *	PMIC*	SMIC <sub>50</sub> *	PMIC*	SMIC <sub>50</sub> *	PMIC*	SMIC <sub>50</sub> *	PMIC*	SMIC <sub>50</sub> *	PMIC*	SMIC <sub>50</sub> *	PMIC*	SMIC <sub>50</sub> *
AMB.	8	31.25	2	1000	1	250	2	250	2	500	0.5	62.5	0.5	>1000	2	1000
NYS.	4.6	1156	4.6	2312	4.6	144.5	9.25	2312	4.6	1156	1.15	144.5	4.6	4625	2.3	578
SRZ.	>16	2000	0.5	>4000	>16	2000	>16	>4000	4	500	>16	4000	>16	2000	0.5	500
MIZ.	16	62.5	0.5	>4000	4	1000	8	125	4	1000	0.125	62.5	2	125	4	500
ECZ.	16	62.5	0.25	>4000	4	1000	4	125	16	1000	0.125	62.5	2	1000	1	125
FLZ.	64	>2000	16	>2000	32	>2000	64	>2000	64	>2000	4	>2000	4	>2000	64	>2000
KTZ.	16	4000	2	>4000	16	250	4	>4000	16	>4000	0.25	31.25	16	2000	4	1000
TRB.	64	8000	2	4000	1	125	16	2000	16	1000	1	62.5	1	125	4	500
N.B	All conc. are by µg/ml															
	PMIC*: Is the minimum drug conc. cause complete inhibition of planktonic candida.															
	SMIC <sub>50</sub> **: Is the minimum drug conc. cause a 50% inhibition of the sessile candida (candida biofilm).															



**Fig.3: Scanning electron micrograph showing the biofilm of *C. albicans* on polycarbonate membrane filters:**

*I.* cultivated in nystatin at sub-inhibitory concentration of the planktonic cells (4.5 µg/ml) (a) 6 hrs. (Mag. 5000X); (b) 12 hrs. (Mag. 5000X); (c) 24 hrs. (Mag. 5000X); (d) 48 hrs. (Mag. 5000X); *II.* Cultivated in nystatin concentration at 2312 µg/ml after (e) 24-hrs *C.albicans* biofilm (Mag. 8000X) and *III.* cultivated in nystatin at subinhibitory concentration of the planktonic cells (4.5 µg/ml) (f) 24-h biofilm cells (Mag. 5000X).

### CONCLUSION

We have shown the effect of some antifungal drugs on the preformed *candida albicans* biofilms and the antifungal penetration through different candida biofilms.

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