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ORIGINAL ARTICLE

Biofilm formation and drug susceptibility of biofilm *Candida* spp. clinically isolated from nasopharyngeal cancer patients in Vietnam

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ABSTRACT

Background and Objectives: The biofilm formation has been widely recognized as one of the main mechanisms of antimicrobial resistance development in microorganisms. However, few studies are focusing on this phenomenon in *Candida* spp. in clinical settings, especially on immuno-compromised patients.

Materials and Methods: In this study, both the rate of biofilm formation in those patients and its drug susceptibility in initial and mature biofilm were assessed using crystal violet assay and dilution method.

Results: The results demonstrated that the biofilm formation rate was similar between albicans and non-albicans *Candida*. However, the biofilm formation capacity was more pronounced in non-albicans *Candida*, especially, *C. glabrata*. As expected, there was a significant relationship between biofilm formation and drug resistance. In addition, our study reconfirmed that the age of high concentration of antifungal agents only affected *Candida* before its biofilm formation regardless of its biofilm formation capacity. In the contrary, once the biofilm was formed even elevated drug concentrations did not show sufficient efficacy, highlighting a need for high dosage at the early stage of treatment for those patients.

Conclusion: The results of this study highlighted the importance of using appropriate antifungal agents for *Candida* treatment before the formation of biofilm.

Keywords: *Candida*; Drug resistance; Fungal; Biofilms

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INTRODUCTION

Candida, a common type of yeast-like fungi, plays an important and complicated role in the world of microbiology and global health (1, 2). With over 150 species in nature, *Candida* are ubiquitous and frequently found in different environments, including lands, air, plants, animals, and humans (3). They exist as both harmless commensals and opportunistic pathogens, depending on hosts and their immune systems. Indeed, health issues in humans related to *Candida* spp. arise when *Candida*'s normal homeostasis is disrupted leading to overgrowth of this fungi. Several specific triggers worsen the *Candida*'s unequilibrium within the human microbiota, causing infections (3). They are namely the use of antibiotics, the decline of immune systems, changes in hormones, and invasive medical interventions. The severity of *Candida* infections varies widely from superficial skin and mucosal to severe systematic ones (4). Especially, under such conditions, *Candida* spp. can undergo an important transformation, from the single-cell and benign form into a multicellular-organized and malign community, which is defined as the formation of biofilm (5).

Biofilm, a phenomenon in which communities of microorganisms organize themselves in a self-produced matrix, has incessantly drawn the attention of scientists to the fields of microbiology, medicine, and environmental science for several reasons (6). On the one hand, in this state, bacteria, fungi, or a combination thereof, possess a potent capacity to adhere to the surfaces of their host and contaminate a larger range of environments, from natural ones (rivers, lands, oceans...) to artificial equipments, such as medical devices or water pipelines (6, 7). On the other hand, its important resistance capacity to conventional antimicrobial agents is one of the most striking and clinically significant features of biofilm (8). Biofilm-linked antimicrobial resistance is a dynamic and multifaceted phenomenon, which arises from a complex interface of biological, chemical, and physical parameters (9). In addition, microorganisms in their biofilm state exhibit a collective behavior that can make them approximately 5-8 times more resistant to antimicrobial agents (10). Several key factors can explain this resistance, including the stability of their extracellular matrix, the reduction in metabolic activities, adaptation in genetics, quorum sensing, and a higher host immune evasion capacity (10-12). For all the aforementioned reasons, biofilm is widely recognized as a major challenge in the fight against antibiotic resistance and the development of appropriate and effective strategies to prevent and/or reduce biofilm-related infections is in high demand (13, 14). If the biofilm formation is reduced, not only treatment outcomes are improved, but also the safety of medical devices is better ensured (15). Furthermore, an important and susceptible source of the drug resistance phenomenon in agriculture and nature may be minimized.

In the context of *Candida*, biofilm is particularly concerned due to its tight connection with a wide range of human infections' prognosis, including oral thrush, vaginal infections, and serious systematic ones, especially in the case of immunocompromised patients (16, 17). In particular, for individuals with weakened immune systems and/or taking antifungal drugs regularly, the risk of disrupting the equilibrium of oral microbiota is importantly increased and leads to the formation of oral *Candida* biofilms, which represent an important clinical concern for physicians (18). Understanding the formation, structure, and resilience of these biofilms is vital for developing effective strategies for prevention and treatment, ultimately improving the quality of life for those affected by oral thrush and related conditions (19).

In this research, our primary focus was to assess the biofilm-forming capabilities of various *Candida* species clinically isolated from Vietnamese cancer patients. Additionally, we aimed to evaluate the susceptibility of these *Candida* isolates to commonly used antifungal agents, including fluconazole, clotrimazole, miconazole, and nystatin. The study yielded noteworthy insights into the biofilm formation patterns and antifungal resistance of *Candida* species, with a special focus on *Candida glabrata* – the species with the highest biofilm-formation capacity.

Furthermore, this research also investigated the rate of drug resistance related to biofilm formation that may help practitioners with choosing appropriate treatment regimens in clinical settings.

MATERIALS AND METHODS

Materials. RPMI-1640 w/L-Glutamine, Phenol red, 0.2% Glucose, and 0.165 moles per litre MOPS buffer w/o Sodium bicarbonate were purchased from HiMedia (Kelton, PA, USA). Fluconazole, clotrimazole, miconazole, and nystatin powder were provided

by an EU-GMP pharmaceutical factory in Vietnam with a purity superior to 99.99% (Boston Pharma, Hochiminh City, Vietnam). Crystal violet solution 1% was purchased from Merck (NY, USA).

Candida strains/ Cell culture of Candida strains. *Candida* species were isolated from wet swaps of 76 cancer patients by CHROMagar™ *Candida* agar plates and incubated at 37°C for 48 hours identified using PCR-RFLP in our previous study (20). They were then freeze-thawed for subsequent assessment of biological properties. In this research, colonies of the *Candida* isolates were streaked and allowed to grow overnight in YPG broth (Yeast extract 10 g/L, Peptone from casein 15 g/L, Glucose 20 g/L). *Candida* cells were then harvested and washed three times with phosphate-buffered saline (PBS) solution to remove any extraneous materials or media components. *Candida* cells were subsequently dispersed in RPMI-1640 to obtain a suspension of 10^5 CFU/mL.

Biofilm production assay. The assessment of biofilm formation (BF) capacity involved several steps to quantify the adherence and biofilm growth of *Candida* isolates. The procedure was designed to evaluate the capacity of these isolates to form biofilms, which appeared as complex, multicellular communities of microorganisms embedded in a self-produced matrix. In this assay, 100 µL of the *Candida* suspension was inoculated into 96-well polystyrene plates without coating nor surface modifications that might affect biofilm adherence. The plates were incubated for 48 hours at 37°C in a humidified environment. After the incubation period, the plates were manually washed three times with PBS solution to remove any non-adhered and biofilm-formed *Candida* cells. The extent of biofilm formation was measured semi-quantitatively by an ELISA reader (Azure, USA). In a 96-well plate, 1% crystal violet (CV) solution was added to each well. The plates were then incubated in the dark at 37°C for one hour. Subsequently, the plates were washed manually with PBS to remove excess dye completely. Biofilm formation was assessed by measuring the optical density (OD) at 595 nm using an ELISA reader. Changes in colorimetric values at this wavelength are indicative of the density and viability of the biofilm. The strains were categorized into groups based on their biofilm-forming abilities: strong biofilm formers ($OD_{595} > 0.32$), moderate ($OD_{595} = 0.12 - 0.32$), weak ($OD_{595} < 0.12$), and strains that did not form biofilm ($OD_{595} \leq OD_{595}$ of the positive control). *Candida albicans ATCC10231* was used as a reference strain in each experiment. This allowed forcomparison of biofilm formation between the test isolates and the known standard. All biofilm measurements were triplicated for each sample.

Effect of antifungal agents on biofilm cells and planktonic cells. The inclusion of a serial range of antifungal solutions (nystatin, fluconazole, clotrimazole, and miconazole) in the biofilm formation assay permitted a comprehensive evaluation of the activity of these antifungal drugs on both biofilm formation and mature biofilms.

Investigation of antifungal agents' activity on the biofilm formation of *Candida* spp. *Candida* cells with biofilm-forming capacity obtained were prepared and plated onto 96-well plates as described in the biofilm formation test. A concentration range for each antifungal agent was prepared as follows 16 - 0.03124 µg/mL for nystatin, miconazole and clotrimazole, 128-0.25 µg/mL for fluconazole. Each concentration was added to wells in 96-well plates. The plates were then incubated for 48 hours and the biofilm-forming capacity was assessed as described above to determine Biofilm Inhibitory Concentration (BICf). Additionally, the minimum inhibitory concentration (MIC) of those planktonic cells was also studied by measuring the optical density (OD) at 530 nm.

Investigation of antifungal agents' activity on mature biofilms. To investigate the effect of common antifungal agents on mature biofilms, the plates with *Candida* suspension were incubated for 48 hours, allowing the formation of mature biofilm. After the incubation time, unattached cells were removed and wells were washed three times with PBS. Then, a serial range of antifungal solutions as described in the above section was added. Afterwards, the wells were re-incubated for 48 hours. To study the effect of antifungal agents on mature biofilms, the reverse to planktonic cells (measured at OD_{530}) and the remaining biofilm (measured at OD_{595}) were determined and presented as BICp.

Ethical aspects. All sampling procedures employed in the current project were reviewed and approved by the ethics committee of the Ho Chi Minh

City Oncological Hospital as described in our previous publication (20).

RESULTS

Biofilm-forming capacity exhibited by clinical *Candida* spp. isolates. As described in our previous study, the proportion of each *Candida* spp. was identified as: *C. albicans* (63.16%), *C. krusei* (5.25%), *C. glabrata* (10.53%), *C. tropicalis* (10.53%) and *C. tropicalis* (10.53%). The biofilm formation capacity of these isolates was assessed and presented. Fig. 1A and Table 1 summarizes the proportion as well as the biofilm formation capacity of each types of *Candida* spp.

Amongst tested clinical *Candida* spp., *C. albicans* was expectedly the most dominant species in terms of quantity (48/76 isolated strains (63.16%)) and presented the highest rate of biofilm-forming strains (41/66 strains with biofilm-formation capacity (62.12%)). Indeed, a total of 41/48 (85.4%) *C. albicans* revealed an ability to produce biofilm. Nevertheless, the majority of them presented a weak biofilm-forming capacity (36/48 strains (75%)), only 3/48 (6.25%) strains had a strong biofilm capacity and 2/48 (4.17%) strains were observed with moderate biofilm-forming extent.

For non-*albicans Candida* species including *C. glabrata, C. krusei, C. tropicalis* and *C. parapsilopsis*, the prevalence in clinical settings was relatively similar. The results showed a similarity of biofilm-forming capacity for non-*albicans Candida* and *C. albicans* isolates (25/28 strains (89.29%) and 41/48 isolates (85.42%) respectively). However, the rate of strong and moderate biofilm-formation capacity was shown to be higher for non-*albicans Candida* (9/28 isolates (32.14%)) than *C. albicans* ones (5/48 isolates (10.41%) compared to 3/48 (6.25%), respectively). In addition, the biofilm-formation capacity of non-*albicans Candida* appeared to be highly varied amongst the species.

As shown in the Fig. 1B, *C. glabrata* isolates demonstrated the strongest biofilm-formation capacity whereby 62.5% (5/8 strains) of the tested isolates showed either a strong or moderate biofilm-formation capacity. In contrast, the capacity of other non-albicans *Candida* was significantly lower. Indeed, this rate was 25% (1/4), 25% (2/8), and 12.5% (1/8) for *C. krusei*, *C. tropicalis* and *C. parapsilopsis*, respectively. This result was contradictory to another 2019 study in France, in which, *C. albicans* isolates were shown to be the most significant biofilm producers and *C. glabrata* were the weakest species (21). Additionally, the 2011 study in Spain reported no significant difference in biofilm-formation capacity in all tested *Candida* strains (22).

Based on the obtained results, 14 clinical strains with strong or moderate biofilm-formation capacity, namely 8XT, 6XD, 6THCa, 2b, 2a, 14a, 10T, 116, 69, 68, 66, 54, 53, 16, and the standard strain *C. albicans* ATCC 10231 were selected for further investigation.

Investigation of antifungal agents' activity on the biofilm formation of *Candida* spp. The minimum inhibitory concentration for inhibiting biofilm formation (BICf) of widely used antifungal agents for *Candida* treatment in Vietnam was determined. Furthermore, Table 2 represents the comparision between the BICf and the resistance rate of the isolates.

For Nystatin: 15 tested strains all belonged to the intermediate group and were sensitive to nystatin according to results published in a previous study (20). As there was no current regulation on the nystatin breakpoint, the MIC_{50} value was used to determine the effect of nystatin on clinical fungal isolates. The results showed that for strains sensitive to nystatin, the ratio was MBC/MIC = 4, while for strains with intermediate sensitivity, this value was 2. Furthermore, there was no observable difference between *C. albicans* and *C. non albicans.*

Table 1. The biofilm-formation capacity of clinical *Candida* spp. isolates.

Order	Type of *Candida*	Proportion	Strong biofilm formation	Moderate biofilm formation	Weak biofilm formation	No biofilm formation
1	*C. parapsilosis*	8/76 (10.53%)	1/8	0/8	5/8	2/8
2	*C. glabrata*	8/76 (10.53%)	5/8	0/8	3/8	0/8
3	*C. krusei*	4/76 (5.25%)	0/4	1/4	2/4	1/4
4	*C. tropicalis*	8/76 (10.53%)	1/8	1/8	6/8	0/8
5	*C. albicans*	48/76 (63.16%)	3/48	2/48	36/48	7/48

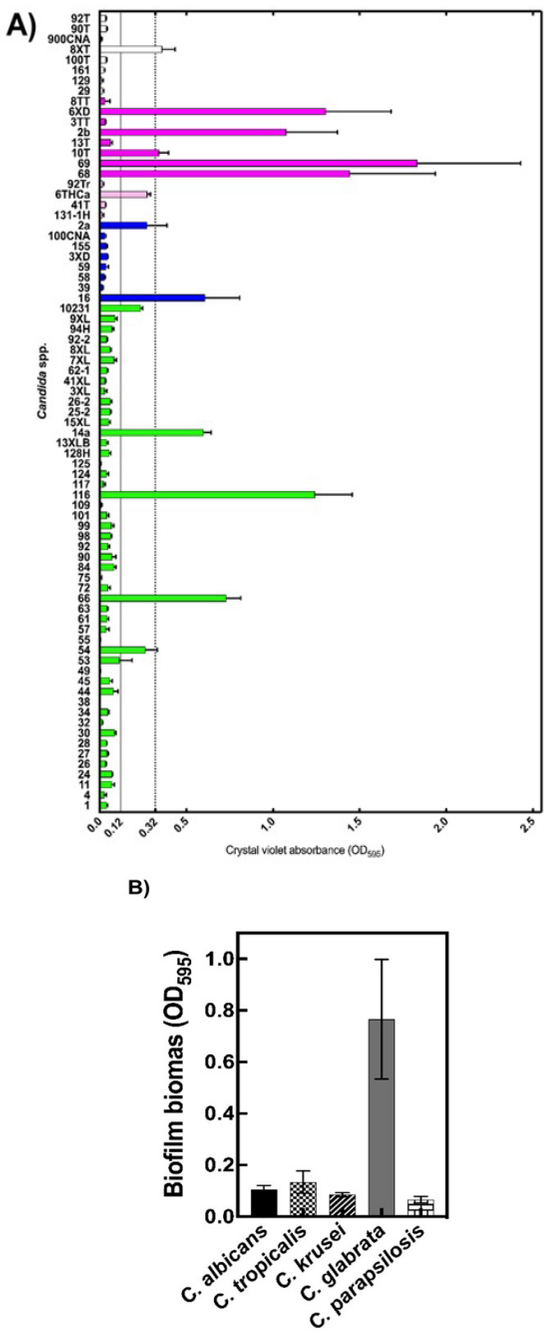

Fig. 1. The biofilm-forming capacity of clinical *Candida* spp. isolates. A) Total biomass of biofilm formation presented by mean values and standard deviations of crystal violet absorbance at 595 nm (OD_{595}); B) The biofilm formation of each kind of *Candida* spp.

Table 2. Biofilm formation capacity of *Candida* spp. in presence of antifungal agents.

Sample	Type of *Candida*	Nystatin			Fluconazole			Clotrimazole			Miconazole		
		BICf (µg/mL)	MIC (µg/mL)	Drug susceptibility	BICf (µg/mL)	MIC (µg/mL)	Drug susceptibility	BICf (µg/mL)	MIC (µg/mL)	Drug susceptibility	BICf (µg/mL)	MIC (µg/mL)	Drug susceptibility
ATCC 10231	*C. albicans*	4	1	S	32	2	S	8	0.125	S	8	0.5	S
53	*C. albicans*	4	1	S	>128	>128	R	16	0.5	I	>16	2	S
54	*C. albicans*	4	1	S	16	1	R	8	0.5	I	>16	4	R
66	*C. albicans*	16	4	S	16	1	R	8	0.5	R	>16	4	I
116	*C. albicans*	2	0.5	S	64	4	R	16	0.5	I	>16	4	I
14a	*C. albicans*	2	0.5	S	16	1	S	8	0.125	S	8	0.5	S
16	*C. tropicalis*	16	8	I	>128	>128	R	16	2	R	>16	16	R
2a	*C. tropicalis*	4	2	S	>128	>128	R	>16	>16	R	>16	>16	R
6THCa	*C. krusei*	8	2	S	128	4	I	16	0.5	S	16	4	I
68	*C. glabrata*	8	4	I	>128	>128	R	>16	16	R	>16	>16	R
69	*C. glabrata*	8	2	S	32	4	S	>16	2	R	16	1	I
2b	*C. glabrata*	16	8	I	>128	>128	R	>16	>16	R	>16	>16	R
10T	*C. glabrata*	8	2	S	>128	>128	R	8	0.25	S	>16	8	R
6XD	*C. glabrata*	4	1	S	64	8	R	>16	1	I	>16	8	I
RXT	*C. parapsilosis*	16	2	S	32	4	S	8	0.125	S	8	2	I

For fluconazole, the most widely oral antifungal agent in clinical settings, most strains were capable of forming biofilm with moderate sensitivity or resistance to fluconazole. Only 3/14 strains were shown to be sensitive to fluconazole. MBC and MIC survey results indicated that for susceptible strains, the ratio was MBC/MIC = 8-16, while for intermediate and resistant strains, this value was in a range of 8-32, or higher (in case MBC and MIC are both superior to128 µg/mL).

In the case of clotrimazole, for susceptible strains, the MBC/MIC value varied from 32 to 64, while it was MBC/MIC = 8-32 or cannot be determined (in case MBC and MIC values were both greater than 16 µg/mL) for intermediate and resistant strains.

For miconazole, in susceptible strains, the MBC/MIC value was 16 which was much higher than that of intermediate and resistant strains (MBC/MIC = 2-4 or cannot be determined because MBC and MIC are both greater than 16 µg/mL).

In addition, for all tested drugs, the BICf value was higher than that of MIC.

Effect of antifungal agents on biofilm dispersion and planktonic cells. Measuring biofilm dispersion involves assessing the ability of microorganisms within a biofilm to detach or disperse from the biofilm structure and return to a planktonic (free-floating) state. Biofilm dispersion can have significant implications for both microbial communities and various applications, such as medicine and industry (21). Antifungal agents have a harder time affecting fungal cells detached from biofilms than planktonic cells (22). The percentage of fungal cell loss from preformed biofilm is significantly lower than that of fungal cells in the same concentration in a liquid environment (planktonic cells) for all strains. In other words, the drug MIC value for fungi released from biofilm (biofilm dispersion) is significantly higher than that of floating ones (planktonic cells) (Table 3).

For *Candida albicans*, biofilm dispersion was shown to be more resistant to antibiotics than planktonic cells. For example, the MIC value was increased 4-8 times for nystatin. Especially, in the case of azole agents, this ratio increased dramatically (up to 128 times), for example, for the strain *C. albicans* 66, the fluconazole value was 1 µg/mL on planktonic cells while it was up to 128 µg/mL on cells released from biofilm.

For non-albicans *Candida*, 4/5 *C. glabrata* strains showed a strong ability to produce biofilm. As a result, the MIC of biofilm dispersion reached the highest concentration in all of our tests. Indeed, this value was 16 µg/mL for nystatin, clotrimazole, miconazole and superior to 128 µg/mL for fluconazole. This result indicated that *C. glabrata* was not only naturally resistant to azoles but this trend also occurred with nystatin, suggesting a threatening situation in which, biofilm dispersion is resistant to antifungal agents. For other species of *Candida* spp. the MICs of all tested antifungal agents increased importantly and were close to the highest concentration tested (16 µg/mL for nystatin, clotrimazole, miconazole being 16 ug/mL and 128 µg/mL for fluconazole).

Investigation of antifungal agents' activity on mature biofilms. The antifungal activities of four drugs were tested on mature biofilm in order to evaluate the role of biofilm in drug resistance development (Table 4).

Table 3. MIC (µg/mL) of planktonic cells dispersed from biofilm.

Sample	Type of *Candida*	Nystatin MIC (µg/mL)	Fluconazole MIC (µg/mL)	Clotrimazole MIC (µg/mL)	Miconazole MIC (µg/mL)
ATCC 10231	*C. albicans*	4	>128	1	8
53	*C. albicans*	4	32	16	4
54	*C. albicans*	4	>128	>16	>16
66	*C. albicans*	> 16	128	>16	>16
116	*C. albicans*	4	128	>16	>16
14a	*C. albicans*	4	32	16	16
16	*C. tropicalis*	>16	>128	8	>16
2a	*C. tropicalis*	8	>128	>16	>16
6THCa	*C. krusei*	16	>128	>16	>16
68	*C. glabrata*	16	>128	>16	>16
69	*C. glabrata*	16	64	8	8
2b	*C. glabrata*	16	>128	>16	>16
10T	*C. glabrata*	16	>128	16	>16
6XD	*C. glabrata*	4	128	>16	>16
8XT	*C. parapsilopsis*	16	64	16	16

Table 4. Antifungal drugs' activities on mature biofilm (BICp).

Sample	Type of *Candida*	Nystatin (µg/mL)	Fluconazole (µg/mL)	Clotrimazole (µg/mL)	Miconazole (µg/mL)
ATCC 10231	*C. albicans*	4	>128	>16	>16
53	*C. albicans*	4	>128	>16	>16
54	*C. albicans*	4	>128	>16	>16
66	*C. albicans*	16	>128	>16	>16
116	*C. albicans*	2	>128	>16	>16
14a	*C. albicans*	4	128	>16	>16
16	*C. tropicalis*	>16	>128	>16	>16
2a	*C. tropicalis*	16	>128	>16	>16
6THCa	*C. krusei*	8	>128	>16	>16
68	*C. glabrata*	16	>128	>16	>16
69	*C. glabrata*	8	>128	>16	>16
2b	*C. glabrata*	16	>128	>16	>16
10T	*C. glabrata*	16	>128	>16	>16
6XD	*C. glabrata*	4	>128	>16	>16
8XT	*C. parapsilopsis*	>16	>128	>16	>16

For azole antifungal agents, a complete resistance was unexpectedly observed for all tested clinical strains, as reflected by a BICp above 128 µg/mL for fluconazole and above 16 µg/mL for clotrimazole and miconazole.

For nystatin, a great increase in BICp compared to BICf and MIC was recorded. Nevertheless, this increased rate was lower than that of the azole group. Furthermore, the comparison with BICp and initial MIC of each antifungal agent for all tested strains indicated that this increased rate seemed not to be dependent on the initial resistance of the clinical strain but relied mainly on the formation of biofilm. This finding further confirmed the important role of biofilm formation as a key factor of drug resistance development in clinical fungal strains. In addition, the difference in nystatin and azole drugs may be explained by a better cellular penetration of nystatin compared to azole ones (23, 24).

DISCUSSION

Previous reports have emphasised the significant role of biofilms in the pathobiology of *Candida* infections, leading to treatment failure (21). In litterature, the formation of *C. albicans* biofilms was also determined as the first step in triggering the release of fungal cells from within the biofilm structure (22, 24). These surviving cells are able to withstand high concentrations of antifungal agents and then relocate to form new biofilms upon the complete removal of antifungal agents (6, 24). In addition, an increasing number of immuno-compromised diseases namely oncology, diabetes, and COVID-19 require extensive usage of medical devices for symptom mitigation and cure purposes. This in turn facilitates conducive conditions for biofilm formation on these devices, leading to resistance to fungal cells and cross-infection between patients (25-27). This urgent need requires an investigational study on biofilm formation and its impacts on the extent of resistance susceptibility of fungi at different biofilm-forming stages.

In this study, the ability of biofilm formation in fungal strains extracted from the oral lesions of nasopharyngeal cancer patients was studied, which was the following part of a previous publication of our group on its prevalence and drug susceptibility (20). The results of the current study showed that less than a quarter (14/76 strains) of fungal strains could produce biofilm at a moderate to high level, compared with the control strain ATCC 10231. This rate is low compared to a study in Iran in which there were 60 strains sampled. In that study, the fungal strains extracted from the head, face, and neck of cancer patients had a high ability to produce biofilm, of which 48/60 strains had a higher biofilm mass than strain ATCC 10231 (28). However, our results presented a possible

correlation between the ability to produce biofilm and different kinds of *Candida* species. This correlation was consistent to other studies in litterature (29).

In addition, our findings presented *Candida glabrata* as the most likely species to produce biofilm, which contrasts with other publications on similar groups of surveyed patients. For instance, a study in Iran indicated *Candida tropicalis* to be the species with the highest ability to produce biofilm (28). Another study in India pointed out that *Candida albicans* species possess the highest ability to create biofilm. However, this study was performed on *Candida* strains extracted from patients with candidemia and HIV infection (29). The results suggested that the biofilm formation of *Candida* species is significantly depenant on sample origins, especially in terms of type of cancer and the health state of patients. In our study, nasopharyngeal cancer patients, taking daily antifungal agents as a prevention method, with a health state dramatically reduced due to malnutrition, and patients with catheler implant were the main subjects. This dependance would be further investigated by enlarging and comparing the same issue but for different kinds of cancer.

Analysing our and Iran's research results, there was no evidence showing a positive correlation between the susceptibility level of fungi and the ability to produce biofilm (28). Within the *Candida albicans* group, only one out of five fluconazole-susceptible strains was capable of producing biofilm whereas all five strains were susceptible to nystatin with an even distribution of R and I with clotrimazole and miconazole. Amongst the non-*albicans Candida* group, the strains are naturally resistant to the azole group, so it is impossible to conclude the linkage between susceptibility level and capacity of biofilm formation.

Furthermore, the impact of antifungal agents on two stages of the biofilm cycle was also investigated, including the initial biofilm formation, which starts with fungal cells attaching to a polystyrene surface, and the final stage of the cycle, where the biofilm structure reactivates fungal cells into the surrounding environment. In the first experiment, fungal cells were exposed to antifungal agents from the beginning. This allowed for projecting the effective concentrations of antifungal agents that could prevent fungal cells from adhering to culture wells, thereby inhibiting biofilm formation at the well bottom (BIC). This is because, as soon as the cells attach to the well bottom, the fungi immediately produce nutrients to protect the cells, making the attachment more secured, and hence the BIC concentration is always higher than the MIC concentration of the same test compound. Depending on the resistance level of the *Candida* spp. strain, the BIC/MIC ratio varies, with a higher resistance leading to a lower ratio. Azole inhibits ergosterol synthesis by interacting with the enzyme lanosterol demethylase, which then converts lanosterol to ergosterol in the fungal cell membrane. In the initial phase of biofilm formation, reflux pumps play an important role in initiating the attachment process. Therefore, azole does not have a clear effect at this step.

In the second experiment related to biofilm, the ability to regenerate the number of fungal cells in the liquid environment from biofilm under the influence of antifungal agents was studied. The results showed that in the fungal structure of the biofilm, fungal cells are tightly encapsulated by extracellular matrix (ECM). Therefore, antifungal agents have difficulty penetrating this structure to kill the fungal cells, even at concentrations much higher than the MIC. This results in a release of fungal cells back into the larger environment, and these cells can withstand the effects of antifungal agents better than primitive free-floating fungal cells. The results show that the MIC of the released cells from the biofilm is much higher than the MIC of the primitive free-floating fungal cells. Subsequently, the ability of antifungal agents to destroy preformed biofilms was also studied. This information can suggest which antifungal agents should be used in cases involving artificial materials in treatment, such as patients requiring ventilators, feeding tubes, or esophageal stents. However, the results showed that even at relatively high concentrations (128 μg/mL for fluconazole and 16 μg/mL for other azoles), antifungal agents could only destroy about half of the biofilm mass, whereas, at the same concentration, they could almost eliminate the sensitive *Candida* strains.

From these results, it is evident that higher doses than usual are necessary if the formation of *Candida* biofilms is detected in patients, especially in immune-compromised patients such as cancer patients according to this study.

CONCLUSION

This study successfully revealed the rate of biofilm

formation in clinical fungal strains as well as its susceptibility to common antifungal agents and finally highlighted its important role in the development of drug resistance in immuno-compromised patients. It also emphasized the need to assess the biofilm formation capacity of these pathogens which may help healthcare physicians to apply appropriate treatment strategies. Further studies on the medical profile of such patients to establish a potential risk assessment scoring will be conducted in due time.

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