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Colonization and Putative Virulence Factors of *Candida* Isolated from the Oral Cavity of Cigarette/ Narghile Smokers and Non-smokers

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Authors' contributions

This work was carried out in collaboration between all authors. Authors AAS and NDO designed the study, wrote the protocol and the first draft of the manuscript. All authors managed literature searches and the analyses of the study, read and approved the final manuscript.

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Opinion Article

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ABSTRACT

Aims: This study aimed to investigate colonization, molecular detection, and virulence characteristics of *Candida* species isolated from young adults who smoke cigarettes and Narghile.
Methodology: A total of 238 young males and females which were recruited from the Jordan university students over the period 2013-2014. The participants were divided into 3 groups; non-smokers, cigarette smokers and Narghile smokers. Oral swabs were sampled from tong dorsum and jugal mucosa using sterile cotton swabs pre-moistened with 0.9% saline. Samples were cultured for *Candida* species and their growth and virulence factors were identified using microbiological culture methods, polymerase chain reaction, and random amplified polymorphic DNA.
Results: A total of 30 (12.8%) of *Candida* species isolates were recovered. *C. albicans* was the most commonly isolated species. There was no significant difference in the production of

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proteinase, phospholipase and hemolysin between 17 *C. albicans* and 7 *C. dubliniensis* isolates from the 3 participating groups. Three major genotypes profiles among *C. albicans* were found, and 10/17 (58.8%) of *C. albicans* isolates belong to the major genotype group A, which showed 4 specific bands with different sizes (2,861, 1,504, 1,284, 815 bp).
Conclusion: This study shows that colonization rate of *Candida* species in the oral cavity was higher but not statistically significant in smokers than non-smokers. Most *C. albicans* isolates from smokers and non-smokers produced similar virulence factors and belonged to three major genotypes.

Keywords: Oral Candida colonization; virulence factors; Narghile and cigarette smokers.

1. INTODUCTION

Oral candidiasis is a common yeast infection observed mostly in the oral cavity of immunocompromised and in association with antibiotic treatment [1], and less frequently developed in healthy individuals, and mostly caused by *C. albicans* [2]. Recent studies reported that tobacoo smoking can be predisposing factor to increase incidence of oral candidiasis [3-4]. It has been demonstrated that cigarette smoke condensate increased *C. albicans* adhesion and growth as well as biofilm formation in association with increasing secretion of proteolytic enzymes particularly aspartyl proteinases [4]. Additionally, it has been reported that *C. albicans* once exposed to cigarette smoke condensate, its transition from blastospore to hyphal form will be increased and express high levels of chitin during hyphal formation [5]. These conditions allow *C. albicans* to adhere better to the gingival fibroblasts, proliferated almost three times more and adapted into hyphae, which contributes for candidal infections in the oral cavity of smokers [5].

Generally, the pathogenicity of *C. albicans* is based on its virulence factors which facilitate invading the host cells and induce transition from budding yeast to pseudohyphal form, which contributes to biofilm formation, adhesion to human epithelial and endothial cells. These features would increase the ability of *Candida* species to secrete hydrolytic enzymes especially proteinases and phospholipases [6].

Since centuries, Narghile (water-pipe) smoking has been a traditional type of smoking in wide areas of Asia and Africa including all Middle East countries. The way of smoking differs from that of cigarette smoking, Narghile smoker inhales through a plastic or leather hose, connected to a bowl of water, in order to draw air over the burning charcoal which is placed on top of perforated aluminum foil, which in turn contains tobacco or maassel; a mixture of tobacco with sweetener and flavoring substances [7]. The increasing popularity of Narghile has affected all generations, most recently more young males and females. Unconventional methods and materials associated with Narghile smoking including the use of flavored tobacco (maassel) which has been probably contributed to its increasing popularity and adverse health consequences [7-8].

Narghile smokers are exposed to toxicants such as nicotine, carbon monoxide and aldehyde compounds which are carcinogenic and hazardous [8]. Furthermore, a new study has reported that Narghile smokers are at higher risk for cardiovascular disease, nicotine/tobacco dependence and cancer [9]. Both Narghile and cigarettes have similar toxicants, and some studies have indicated that Narghile smoking has harmful effects on the oral cavity [10]. However, the health effects of smoking Narghile on oral cavity are still not clearly documented.

Few studies have investigated the association of smoking with candidal putative virulence factors. A recent study observed that there is no difference in the profiles of candidal virulence factors among isolates from smokers and non-smokers, particularly with secretion of aspartyl proteinases, esterase-lipase and hemolysins [11].

This study aimed to compare colonization rates, putative virulence factors and common genotypes among frequently *C. albicans* isolates from the oral cavity of young cigarette/ Narghile smokers and non-smokers.

2. MATERIALS AND METHODS

2.1 Study Populations

In this case-control study, participants were recruited from university students who were invited to an oral health awareness campaigns on campus over the period 2013-2014.

2.1.1 Exclusion criteria of study

All cigarette smoker on a daily basis and a Narghile smoker on a daily/ weekly basis, or a non-smoker, were investigated to be free of any oral lesions, and confirmed that they have no illness or treatment with antibiotics during the last month.

After signing a consent form of participation, oral swabs were sampled from tong dorsum and jugal mucosa using sterile cotton swabs pre-moistened with 0.9% saline by 3 senior dentist students. The study was approved by the Faculty of Medicine and Postgraduate Studies at The University of Jordan, and ethical approval was obtained from the Institutional Ethical Review Boards at the Jordan University Hospital. Additionally, the study has been conducted in full accordance with the World Medical Association Declaration of Helsinki.

All oral samples were transferred immediately to the Microbiology Laboratory/ Faculty of Medicine/ The University of Jordan. Samples were collected in 3 groups of around 80 samples each; group 1 is non-smokers (80), which serves as the control group, group 2 is cigarette smokers (81) and group 3 is Narghile smokers (77).

2.2 Culture and Isolation

All samples were inoculated directly onto Sabouraud dextrose agar plates (SDA, Oxoid, UK) and incubated for 48 hours at 37℃. The culture plates were examined to detect the presence of white to cream growth of yeast-like colonies of *Candida* species. These were first identified by preparing germ tube test in human serum. Second, isolated colonies were subcultured on CHROMagar *Candida* plates and incubated for 48 hours at 37℃. All *Candida* isolates observed on CHROMagar *Candida* medium were identified primarily by colony morphology and pigmentation according to the manufacturer's instructions and as described by Odds and Bernaerts [12]. Optimum color intensity of some *Candida* isolates was recorded after 48 hours of incubation. Reference standard strains of *Candida albicans* (ATCC 10231), *Candida krusei* (ATCC 6258) and *Candida glabrata* (ATCC 90876) were included on plates of CHROMagar *Candida* medium as control strains throughout the study.

2.3 Identification and Genetic Typing of *Candida*

Extraction of DNA from fresh *Candida* growth in Yeast Peptone Dextrose broth (YPD) was performed using Wizard Genomic Purification Kit (Promega, USA) according to manufacturer's instructions. Detection of *C. albicans* and *C. dubliniensis* was determined using primers derived from unique rDNA sequences specifically ITS-1 and ITS-2 regions of rDNA by duplex PCR, and other *Candida* species were detected using three 10-mers (OPA-18, OPE-04, OPE-18) and RAPD PCR with some modifications [13]. The amplification of these sequences was performed in a total volume of 25 µL. Qiagen PCR buffer 10x (2.5 µl) and Qiagen $MgCl_2$ 25 mM (0.375 µl) and Dntps mix 10 mM (2 µl) were added to the mixture. The primer quantities used in the PCR reaction were; (0.5 µl) of *C. Albicans* (CAL) primers and (0.5 µl) of *C. dubliniensis* (CDU) primers.0.25 µl KAPA Taq polymerase and 4 µl of the extracted DNA were added to the reaction mixture. Nuclease free water was added to achieve a final volume of 25 µL of the PCR reaction mixture. PCR conditions were set in a PCR thermocycler with an initial denaturation at 95℃ for 5 minutes, followed by 35 cycles of denaturation at 95℃ for 1 minute, then annealing at 55℃ for 30 seconds, and extension at 72℃ for 1 minute, and a final extension at 72℃ for 10 minutes. The PCR product with a volume of 8 µl was loaded into wells of 2% agarose gel with 1X TBE buffer and was run for 1 hour at 110 volts. A 100- bp DNA ladder was used as the molecular size marker, and then the agarose gel was visualized by UV Transilluminator (UVP) system.

Molecular typing of major *C. albicans* groups was determined using short 10-mers primers by RAPD according to the technique described by Bautista-Muñoz [14]. A positive control of *C. albicans* ATCC 10231 was used.

2.4 Qualitative Determination of Putative Virulence Factors

Detection extracellular production of proteinase was performed as follow: *C. albicans* and *C. dubliniensis* fresh isolates (10 µl suspension of 10^7 yeast cells /ml) were inoculated as spot on the surface of plate containing bovine serum albumin agar (Biolife, Italy). The plates were incubated in humidified conditions at 37℃ for 5 days, and then were flooded with the staining solution (Napthalene Black 10 B dissolved in

methanol-acetic acid) for 15 min. After decolorized of plates for 36 hours using 15 % glacial acetic acid, the diameter of clear zone of proteolysis around *Candida* colony growth were measured an recorded as positive in comparison to the positive *Candida* control strain (*C. albicans* ATCC 10231) [15]. Production of extracellular phospholipase activity was estimated by inoculated the same fresh *Candida* isolates suspension on egg-yolk agar plates and incubation at 37℃ for 48 hours. The production of the phospholipase was determined qualitatively by the presence of the precipitation zone around the *Candida* colonies [15]. Beta-haemolysin production was evaluated using a fresh human blood agar plate and after incubation for 48 h, the presence of distinct translucent halo around the inoculums site, viewed with transmitted light, indicated positive hemolytic activity [15]. All tests were conducted in duplicate using control *Candida* strain as described previously by Shehabi et al. [16].

2.5 Statistical Analysis

Data generated from the study were tabulated as Microsoft Excel sheets and Uploaded to Statistical Package for Social Sciences (IBM SPSS version 20). SPSS was used to calculate frequency and percentage for the categorical data, values of significant differences between the groups and to carry out regression analysis. In all statistical tests, the differences were considered to be statistically significant if *P* value < 0.05.

3. RESULTS

A total of 238 oral samples were collected from the participants, their age ranged between (18-26 years; (mean 21.37±2.05SD). The study included 27 (33.8%) males and 53 (65%) females of non-smokers, 75 (97.4%) males and 2 (2.6%) females of cigarette smokers, and 42 (51.4%) males and 39 (48.1%) females of Narghile smokers. One-way ANOVA showed that there was no significance difference between the control group (non-smokers) and both groups; the cigarette smokers group and the Narghile smokers group. All smokers confirmed that are smoking for at least one-year.

(Table 1), the distribution of *Candida* species isolates from oral cavities of 238 smoker and non-smokers are presented in Table 1. No significant difference was found among the three groups. A total of 30 *Candida* species isolates was recovered, including 17 (56.7%) *C. abicans*, 7 (23.3%) *C. dubliniensis* and 6 (20%) other *Candida spp.* The production of proteinase, phospholipase and hemolysin enzymes of *C. albicans* and *C. dubliniensis* was compared between non-smokers, cigarette smokers and Narghile smokers groups. There was no significant difference according to their qualitative production (P>0.05) (Table 2). Table (3) shows the major genotype profiles among *C. albicans* isolates using RAPD-PCR technique. Group A includes 10 (58.8%) isolates and showed each 4 bands (2,861, 1,504, 1,284, 815 bp) which can be considered the major genotype, followed by group B which includes 4 (23.5%) isolates and separated into each 3 bands (2,861, 1,284, 1,100 bp), and group C includes 3 (17.7%) isolates and each separated into 2 bands (2,861, 1,284 bp).

4. DISCUSSION

This study indicated that colonization of *Candida* species in the oral cavity among Jordanian students who smoked Narghile was (14.8%), and it was slightly higher than in cigarette smokers (11.7%) and nonsmokers (11.3%), however, there was no statistical difference between these 3 groups. *C. albicans* was the most prevalent species (56.7%) among the 3 groups. It is important to note that *C. dubliniensis* is the second most common species (23.7%), although this species was often previously isolated from immunocompromised patients [13,17], but it has been also found in patients with diabetic mellitus and in less rates in the oral cavity of healthy persons [2].

Table 1. *Candida* species colonizing the oral cavities of 238 Narghile and cigarette smokers and non-smokers (mean age 21.37 year)

***Candida* species**	**No. (%) non-smokers**	**No. (%) Narghile smokers ***	**No. (%) cigarette smokers***	**Total no. (%) isolates**
C. albicans	5 (6.3)	8 (9.9)	4 (5.2)	17(56.7)
C. dubliniensis	2 (2.5)	2 (2.5)	3 (3.9)	7 (23.3)
C. parapsiolosis	0	2 (2.5)	1 (1.3)	3(10)
Candida glabrata	1 (2.5)	0	1 (1.3)	2(6.7)
C. krusei	1 (1.3)	0	0	1(3.3)
Total no. (%)	9 /80(11.3) *****	12/81 (14.8) *****	9 /77(11.7) *****	30 (100)

*P= >0.5 among the 3 groups * All smokers of Narghile and cigarette have smoked at least for one-year*

Table 2. Distribution of proteinase, phospholipase and hemolysin production among *Candida albicans* and *C. dubliniensis isolates**

Candida isolates (no.)	No. (%) isolates positive for		
	Proteinase	**Phospholipase**	**Hemolysin**
C. albicans (17)	11/17 (65)	9/17 (53)	11/17(65)
C. dubliniensis (7)	5/7 (71)	2 /7(28)	3/7(43)

** Production of virulence factors were not significant (P= >0.5) between the two Candida species*

Table 3. Major genotype patterns of *C. albicans* isolates using RAPD PCR

C. albicans	No. (%) of isolates	No. of bands	Band size(bp)
Group A	10 (58.8)*	4	2,861, 1,504, 1,284, 815
Group B	4 (23.5)	3	2,861, 1,284, 1,100
Group C	3 (17.7)	2	2,861, 1,284
Total no. (%)	17 (100)		

** P= <0.5*

One of the limitations of this study was the unequal numbers of males and females represented in the cigarette and Narghile smokers groups. This limitation is attributed to decreased popularity of cigarette smoking among female students who prefer Narghile to cigarettes due to many reasons; one of which is the social acceptance and tolerance by the community [7]. The relatively low prevalence of oral *Candida* species carriage among participants (30; 12.8%) may be due to the fact that all samples were obtained from healthy young university student.

Several studies from our region and other countries reported that tobacco smoking either alone or in association with other factors, were associated with increased oral candidal colonization especially in HIV-infected patients [18-19], and it has been demonstrated that *C. albicans* virulence was increased after exposition to cigarette mainstream smoke[20]. Other studies showed clearly as our study that oral candidal carriage in smokers and nonsmokers is the same [21-22].

The present study demonstrated that there is no significant difference in the production of proteinases, phospholipase and hemolysins among *C. albicans* and *C. dubliniensis* isolates from smokers and nonsmokers (Table 3). Both organisms are highly related pathogenic yeast species, but *C. albicans* is far more prevalent in human infection and has been proved to be more pathogenic in a wide range of infection models, particularly in association with production of putative hydrolyses [23-24].

Most *Candida* species isolates from human can secrete three different classes of hydrolases during hyphal growth which allow them active penetration into host cells. These virulence factors can be detected qualitative and quantitative *in vitro* tests [13,16]. In addition, numerous studies have demonstrated that *C. albicans* isolated from oral cavity and other body sites secrete more putative virulence factors *in vitro* than other *Candida* species [16,25-27]. However, certain studies have reported that *Candida* strains isolated from infected body sites and healthy individuals showed an overall high similarity in their potential secretion of virulence factors, suggesting that all commensal strains have the ability to develop as opportunistic pathogens [24-26].

It was also reported that only a significantly higher phospholipase and chondroitinasic activities in *C. albinans* strains were obtained from non-smokers than smokers [12], while the other enzymes activities were the same. Furthermore, one study has suggested that cigarette smoke condensate enhance significantly the secretion of candidal enzymes and adherence to denture surfaces which is associated with higher oral candidal carriage and possible oral candidasis [20].

The present study showed the prevalent of only 3 major genotypes of *C. albicans* in oral cavity of Jordanian students using random amplified polymorphic DNA PCR-assay. The prevalent group A includes (58.8%) of the isolates. A previous study from China, has reported the presence of 4 major genotype of *Candida* groups detected in 112 patients and healthy control [28]. This study confirms previous studies which have demonstrated that RAPD methods performed with different oligonucleotides basically generated consistent patterns, with several shared fragments unique to each *Candida* species [15].

5. CONCLUSION

This study suggests that there is no significant difference between individuals smoking Narghile or cigarettes and non-smokers in relation to colonization with *Candida* species or their secretion of potential virulence factors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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