

Frequency of *Candida* Species in the Oral Cavity of Narcotics and Stimulants Smokers in Isfahan, Using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism Method

Abstract

Background: *Candida* species are a group of coexistent microorganisms in the oral cavity that opportunistically cause disease in vulnerable people, including addicts. This study is aimed at identifying and determining the frequency of *Candida* species in the oral cavity of the addicts to inform the epidemiological panel and to warn to prevent the resistance of the species. **Materials and Methods:** In this study, sampling was done from the oral cavity of 83 addicts who referred to Addiction Treatment Centers in Isfahan, Iran, using moist sterile swab. The presence of yeast on the direct microscope slides of 58 samples was confirmed. To carry out culture and the primary identification, Sabouraud dextrose agar medium with chloramphenicol as well as HiCrome *Candida* agar medium was used. Isolates were identified by polymerase chain reaction (PCR) restriction fragment length polymorphism (using specific primers ITS1-ITS4 and *MSP* I enzyme) and Duplex PCR test (using specific primers CDU1-CDU2/CAL1-CAL2). **Results:** Out of 93 *Candida* isolates which had been isolated from 58 culture-positive samples of the oral cavity, *Candida albicans* (41.93%) were the most prevalent species, followed by *Candida dubliniensis* (24.73%), *Candida glabrata* (21.50%), *Candida kefyr* (8.60%), *Candida tropicalis* (2.15%), and *Candida parapsilosis* (1.07%), respectively. In 33 cases (56.90%), the presence of more than one *Candida* species was observed. **Conclusion:** Compared to the studies conducted on the oral cavity of healthy controls, smoking certain drugs can have a significant effect on the presence and frequency of *Candida* species, particularly *C. dubliniensis* and *C. glabrata*.

Keywords: Addiction, *Candida*, oral cavity, polymerase chain reaction, restriction fragment length polymorphism

Introduction

The human oral cavity is naturally the habitat of microorganisms such as yeast-like *Candida*, which in natural conditions without developing diseases, coexists with other microorganisms present in the flora of this region. Generally, about 25%–75% of people have oral *Candida* flora.^[1-3] The genus *Candida* contains 150 species, among which there are >20 important medical species.^[4] The most yeasts which are commonly separated from the oral cavity are *Candida albicans* (47%–75%), *Candida tropicalis* (7%), *Candida glabrata* (7%), and *Candida parapsilosis*, *Candida krusei*, and *Candida guilliermondii*, each <5%.^[5]

Candida species, like other opportunistic fungi, are capable of causing infection due to the initial physiological and immunologic

host weakness or genetic and underlying factors.^[6] Among the underlying factors which cause these opportunistic diseases are: diabetes, immunodeficiency syndrome, soft tissue injury, loss of natural defensive barriers, dryness and lack of saliva, artificial teeth, smoking cigarettes, and addiction. The infection resulting from *Candida* is called candidiasis, which is one of the most common opportunistic infections of the oral cavity and has several types.^[7,8] One of the important underlying factors which lead to candidiasis is addiction.^[9,10] Addiction to drugs is defined as the condition in which the drug abusers restrict their goals to searching for the drugs, its abuse and enjoying it, as well as continuing all these,

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How to cite this article: Hassani Abharian P, Dehghan P, Hassani-Abharian P, Jabalameli Z. Frequency of *Candida* species in the oral cavity of narcotics and stimulants smokers in Isfahan, using polymerase chain reaction-restriction fragment length polymorphism method. *Adv Biomed Res* 2020;9:30.

Received: 10 February 2020
Revised: 29 April 2020
Accepted: 11 May 2020
Published: 27 July 2020

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Access this article online

Website: www.advbiores.net

DOI: 10.4103/abr.abr_38_20

Quick Response Code:



although their undesirable and troublesome side effects. Indeed, addiction is a chronic, progressive, reversible, and destructive disease that creates many structural and functional changes in the brain and affects all aspects of the drug abusers', their families, and relatives' lives.^[11,12]

Addiction is the result of a series of interactions that arise from drug abuse and its reciprocal internal and external effects on an individual. In this case, drug abuse turns to be an impetus in the individual's life.^[13] Addictive drugs are classified in a variety of ways; one of the most widely-used methods categorizes the drugs into seven groups: drugs, stimulants, hallucinogenic drugs, cannabis or cannabinoids, sedative-hypnotic medications, alcohol family and ultimately inhalable family such as ether and gasoline. Instances of narcotics include opium, sap, burned opium, heroin, heroin crystals, and morphine. Cocaine, crack cocaine, amphetamines, and methamphetamine can also be mentioned as instances of stimulants.^[14] Addictive drugs can be used orally as well as through smoking, inhalation, and injection. Smoking is mostly common for narcotics, stimulants, and cannabinoids family.^[15] Today, addiction is considered as an increasing problem for health and public hygiene due to the prevalence of various complications and infections.^[16,17] The previous studies depicted that oral lesions were more prevalent in drug users compared to the general public.^[10] There is a long and recognized relationship between addiction and an increase in the level of infection, including fungal infections.^[18,19] Concerning the current claims, with weakening the immune system, addiction provides conditions for the development of chronic infections such as oral thrush or candidiasis.^[20,21] A plethora of previous studies indicates that oral candidiasis is one of the most common oral infections, particularly in individuals with weakened immune systems, including addicts, and in some cases, as it becomes resistant to treatment, it creates certain problems.^[20,22] The application of certain substances lead to changes such as Candidiasis, frictional hyperkeratosis, gingivostomatitis, aphthous stomatitis, and herpes simplex, which are normally found in the oral mucosa.^[23]

C. albicans is one of the most prevalent *Candida* species in the oral cavity of healthy and sick individuals and is generally known as a dominant pathogenic species in sensitive hosts.^[22,24] Recently, it has been noted that dissemination of *Candida* through the mouth is one of the reasons for the development of *Candida* disseminated infections in immunocompromised patients.^[25,26] For example, esophageal candidiasis, a common symptom in addicts, especially crack abusers who are infected with AIDS, usually occurs following the dissemination of oral lesions. In Nature *et al.*'s study, the prevalence amount of esophagus' Candidiasis in HIV-positive patients who also used crack cocaine was reported to be 11.6%, whereas such prevalence amount was 1.2% in HIV-positive patients who had not abused crack cocaine. The findings of this study

revealed that this prevalence degree of was reported 3% among the patients who had already had crack cocaine abuse.^[27] The results of Freire-Garabal *et al.*'s experimental studies, which explored the impacts of amphetamine on the advancement of oral candidiasis in rats, indicated a remarkable increase in the number of *C. albicans*, the percentage of developed lesions on the surfaces of the tongue, the percentage of unnatural papillae as well as the presence of mycelium threads in tissue sections of the tongue. While Freire-Garabal *et al.*'s previous studies showed that amphetamine had no statistically significant effects on the latter two.^[28]

Nowadays, the occurrence of systemic candidiasis is considerably increasing and it is recognized that many of these infections are caused by non-*albicans Candida* species, which play an important role, particularly in the development of candidemia and invasive candidiasis.^[29] Non-*albicans candida* agents, in comparison to *C. albicans* ones, are more resistant to common fungal drugs. For example, according to certain studies, *C. glabrata* has intrinsic resistance to the azoles combinations, whereas *Candida dubliniensis* has relative resistance to such combinations.^[30,31] The uncontrolled and unconventional use of antifungal drugs makes the species resistant and increases drug resistance, causing a lot of problems.

Considering the above, since addiction can be considered as a predisposing factor for the development of candidiasis and given the fact that simple and systemic *Candida* infections are caused by commensal species, and many of the species have a variable susceptibility pattern to certain common antifungal drugs.^[32,33] The aim of this study was to determine the frequency of *Candida* species isolated from the oral cavity of drug smokers regarding the type of drug abusing.

Materials and Methods

The present study was an epidemiological descriptive-inferential study that examined 83 narcotic and stimulant drug abusers in Isfahan, Iran. Sampling was done beside the flame by two moist sterile cotton swabs that were simultaneously rotated on the internal surfaces of the whole oral cavity, above and under the tongue. After sampling, one of the swabs was immediately cultured through the sweep-plate method on a Sabouraud dextrose agar medium with chloramphenicol (Merck-Germany), and another swab was used to prepare direct microscope slides stained with Giemsa. The without-yeast samples on direct microscope slides were excluded from the study. Plates were kept at a temperature of 30°C for 48 h, and their growth rate and form of the colonies were examined.

Differentiation of *Candida* species by phenotypic method

After purification, for the initial detection and isolation of the mixed species, the colonies were cultured on HiCrome *Candida* agar (HiMedia, Mumbai, India). The isolates

were identified based on the colors introduced in the manufacturer catalog [Figure 1]. Some of the mixed species were purified again in HiCrome *Candida* agar medium due to color mixing.

Identification of *Candida* species using polymerase chain reaction-restriction fragment length polymorphism method

As the CHROMagar culture medium was not specialized for all *Candida* species, molecular methods were used to confirm the initial detections and to identify the species more accurately.

DNA extraction

In the current research, considering the previous studies, extraction of DNA was carried out via boiling method,^[34] through which a loop of the fresh colony was transferred into 1.5 mL microtubes containing 100 μ l sterile distilled water. After it was mixed, it was heated at 90°C for 20 min in a bain-marie. Subsequently, the microtubes were centrifuged at 10,000 rpm for 10 min and the supernatant containing crude DNA was transferred to 0.2 mL microtubes to be used in molecular tests.

Selection of primers and the target DNA

Replication of the gene responsible for coding ribosomal RNA (rDNA) located in the ITS1-5.8S-ITS2 region was also used in this study. Primers ITS1 and ITS4, with the following sequence, were selected as forward (ITS1) and reverse (ITS4) primers.^[35]

ITS1 5' TCCGTAGGTGAACCTGCGG 3'

ITS4 TCCTCCGCTTATTGATATGC 3'

Polymerase chain reaction process

To carry out the PCR reaction for each sample, a mixture of 12.5 μ l of ready-to-use Master Mix (AMPLIQON, Denmark), 3 μ l of DNA template, 0.5 μ l of both primers (concentrated at 10 pmol), and 8.5 μ l of sterile double distilled water were prepared to obtain 25 μ l final volume per reaction.



Figure 1: Appearance color of *Candida* colonies, isolated from the mouth of drug smoker addicts on CHROMagar *Candida* medium No. 1 shows *Candida tropicalis*, no. 2, 4, 6, 7, 9, 10 and 11 indicate albicans/dubliniensis complex, no. 3, 5 and 8 demonstrate *Candida glabrata* and no. 12 shows a non-albicans *Candida* species

The PCR process during the temperature cycle consists of: an initial denaturation cycle at 94°C for 5 min, followed by 34 consecutive cycles composed of 30 s denaturation at 94°C, annealing at 55°C for 45 s, and extension at 72°C for 1 min. These cycles were followed by a cycle at 72°C for 7 min as the final extension.

The amplicon, which resulted from PCR was loaded in 1.5% Agarose gel and electrophoresis was run on it at 80 volts for 45–60 min. The gel was placed in a special position in the ultraviolet (UV) doc, and the image of the bands was checked on the monitor and recorded [Figure 2a].

Restriction fragment length polymorphism process

At this stage, the *MspI* enzyme was used to cut PCR products. For each 15 μ l reaction, 10 μ l of PCR product, 3 μ l of water, 1.5 μ l of buffer and 0.5 μ l of the *MspI* enzyme were mixed. The microtubes containing the prepared ingredients were incubated at 37°C for 1 h. Finally, the products were loaded in 2% agarose gel.

To run electrophoresis, the bands which had been formed were observed under UV light of the UV doc and were interpreted for each sample considering the scale of the applied marker and the existing standards [Table 1 and Figure 2b].

Duplex-polymerase chain reaction

Concerning the inability of the *MspI* enzyme to differentiate *C. albicans* from *C. dubliniensis*, the differentiation of these two species was done by duplex PCR (dPCR) using specific primers CDU1-CDU2/CAL1-CAL2, and the results were previously reported.^[25]

Data analysis

After conducting all the aforementioned research processes, the data were entered in the SPSS software (SPSS, Inc., Chicago, IL, USA, version 23), and the data were analyzed through descriptive and inferential statistics.

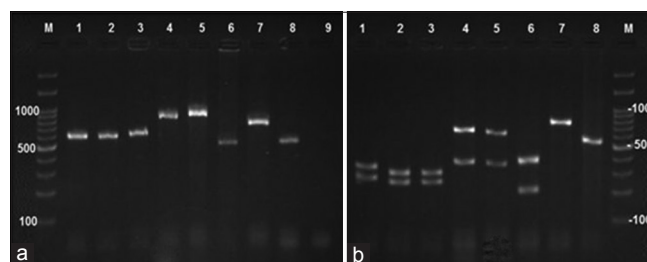


Figure 2: Electrophoresis of polymerase chain reaction and polymerase chain reaction-restriction fragment length polymorphism products of some isolates of this study left (a) Electrophoresis of polymerase chain reaction products of *Candida* isolates using primers ITS 1 and ITS 4 right (b) Electrophoresis of polymerase chain reaction products of *Candida* isolates after endonuclease digestion with *MspI* No. 1-3 show *Candida albicans*, 4 and 5 *Candida glabrata*, 6: *Candida tropicalis*, 7: *Candida kefyr*, 8: *Candida parapsilosis*. 9: indicates the negative control and M denotes 100 bp fragments

Table 1: Results of the polymerase chain reaction-restriction fragment length polymorphism process for all oral isolates of this study

<i>Candida</i> species	Amplicon molecular weight	Molecular weight(s) of restriction product(s)	<i>n</i>
<i>Candida albicans</i>	535	238-279	53
<i>Candida glabrata</i>	871	314-557	20
<i>Candida kefyr</i>	721	721	8
<i>Candida tropicalis</i>	524	184-340	2
<i>Candida parapsilosis</i>	520	520	1

Results

Through the investigation of the colored microscope slides in this study, among 83 samples, the presence of yeast was confirmed on direct microscope slides of 58 samples. The number of colonies from the cultivation of these 58 positive clinical samples varied from 20 to 52,800 on each plate.

Phenotypic investigations

All isolates had the potential to grow in the CHROMagar *Candida* culture medium, and altogether 84 yeast isolates were isolated in this medium, of which 53 isolates were shown with green and were reported as “*albicans/dubliniensis* complex,” 20 isolates were shown with purple and were reported as “*C. glabrata*,” and two isolates were shown with blue and light violet margin and were reported as “*C. tropicalis*.” Moreover, nine samples were recorded as non-*albicans Candida* species and were shown with nonspecific colors in white-to-cream spectrum as well as light pink [Figure 1].

Molecular investigations

The results of the PCR-RFLP experiments confirmed the *Candida* species' identities recognized through phenotypic investigations; furthermore, non-*albicans Candida* samples were divided into eight *Candida kefyr* isolates and one *C. parapsilosis* isolate [Figure 2].

The dPCR assay performed on the samples identified as *C. albicans* proved the presence of 39 *C. albicans* and 23 *C. dubliniensis* isolates.

The frequency and mean of different *Candida* isolates separated from the mouth of drug smokers, and the type of drugs showed in Table 2.

Discussion

Due to the weak and ineffective immune system, addicts are an important group of vulnerable people against opportunistic infections. With the increasing prevalence of addiction in recent years, we have been witnessing the spread of *Candida* infections, especially oral candidiasis, among addicts.^[36,37] Although oral *C. albicans*, particularly in the mouth, still remain at the forefront of *Candida* reports, in recent years, non-*albicans* species have attracted lots of attention due to an increase in emergence, epidemiological

changes, and more resistance.^[38] In this study, out of 93 isolates which were identified, 39 isolates (41.93%) were *C. albicans*, 23 isolates (24.73%) were *C. dubliniensis*, 20 isolates (21.50%) were *C. glabrata*, eight isolates (8.60%) were *C. kefyr*, two isolates (2.15%) were *C. tropicalis* and only one isolate (1.07%) was *C. parapsilosis*. In 33 samples (56.96%) of 58 clinical samples taken, more than one *Candida* species was observed. Considering the remarkable presence of non-*albicans* species, especially the high degree of *C. dubliniensis*, which is a rare species, these quantities seem to be a bit thought-provoking. However, the findings of the current study are in line with Hadzic *et al.*'s study which investigated the frequency of *Candida* species in the mouth of addicts hospitalized in addiction treatment centers in Bosnia and Herzegovina; they reported the frequency of *Candida* species as follows: *C. albicans* (43%), *C. dubliniensis* (23%), and other *Candida* species (34%).^[39] In the aforementioned study, which is the only study found to be very similar to the present study, after differentiating the results in terms of type of drug abuse; in the 19-member group who abused opioids, *C. albicans* (47.4%), *C. krusei* (26.3%), *C. dubliniensis* (15.8%), *C. tropicalis* (5.3%) and *C. glabrata* (5.3%) were observed. Whereas in the 16-member group of alcohol users, *C. glabrata* (19%) had the highest frequency after *C. albicans* (37.5%), followed by *C. tropicalis* and *C. krusei*, each 6.2% as more frequent species. In this group, the frequency of *C. dubliniensis* was reported to be 3.25%. Considering that alcohol abuse in Iran is among the top drug dependence^[40] and most of the participants in this study also had the background of alcohol abuse, the compound results of both groups are expected. The absence of *C. krusei* in the present study and the absence of *C. kefyr* in the study whose findings were compared can be due to differences in the type of drug abused or the regional epidemiological characteristics.

In the study conducted in Saudi Arabia in 2019, Mokeem *et al.* explored the *Candida* flora in the oral cavity of smokers as well as users of waterpipe and electronic cigarettes. While separating the *C. albicans*, *C. tropicalis*, and *C. parapsilosis* species, they reported *C. albicans* as the most prevalent species in all three groups. Although a greater variety of species was reported in the current study probably due to the various used substances and users' hygiene poverty, the presence of all three identified species in both studies can be regarded as a parallel finding in

Table 2: Frequency of *Candida* species isolated from the oral cavity of drug smokers regarding the type of drug

Type of drug	<i>Candida</i> species						Total, <i>n</i> (%)	Mean of colony
	<i>Candida albicans</i> , <i>n</i> (%)	<i>Candida dubliniensis</i> , <i>n</i> (%)	<i>Candida glabrata</i> , <i>n</i> (%)	<i>Candida kefyr</i> , <i>n</i> (%)	<i>Candida tropicalis</i> , <i>n</i> (%)	<i>Candida parapsilosis</i> , <i>n</i> (%)		
Resin	4 (66.7)	-	-	2 (33.3)	-	-	6 (100)	3367
Opium	4 (33.3)	4 (33.3)	3 (25)	1 (8.3)	-	-	12 (100)	1271
Heroin	8 (42.1)	6 (31.6)	3 (15.8)	1 (5.3)	1 (5.3)	-	19 (100)	400
Crack cocaine	3 (30)	5 (50)	2 (20)	-	-	-	10 (100)	10824
Methamphetamine	6 (37.5)	3 (18.7)	4 (25)	2 (12.5)	-	1 (6.25)	16 (100)	2095
Opium and methamphetamine	2 (40)	1 (20)	2 (40)	-	-	-	5 (100)	395
Heroin and methamphetamine	9 (53)	2 (11.8)	5 (29.4)	-	1 (5.9)	-	17 (100)	4274
Crack cocaine and methamphetamine	4 (50)	1 (12.5)	1 (12.5)	2 (25)	-	--	8 (100)	1352

these two studies. Climate conditions and sample size can be among the other effective factors in differences.^[41]

Javaheri *et al.*, conducted a study to identify *Candida* species in smokers' saliva. They reported *C. albicans* (42.10%) as the most prevalent species followed by *C. kefyr* and *C. krusei*. Unlike the current study, they found no isolates of *C. parapsilosis*, *C. glabrata*, and *C. dubliniensis*.^[42] Although this study has been carried out on cigarette smokers, the differences in the type of smoking, geographic climate, and method may explain the inconsistencies.

In a research conducted by Heidarian *et al.*, to identify the *Candida* species isolated from oral lesions of HIV-positive people in Isfahan, *C. albicans* had the highest frequency (44%) followed by *C. glabrata* (26.8%), *C. dubliniensis* (17.1%), *C. kefyr* (4.8%), and *C. Tropicalis* (4.8%).^[43] The results of this study are relatively consistent with the findings of the present study. Differences in values seem reasonable due to the differences in the community and immunological disorder. Probably, narcotic and sexual behavior are one of the factors influencing the variety of frequency distribution of *Candida* species.

Studying a variety of samples of 124 patients with immunodeficiency virus in India, the most frequent of which was oral samples (82 patients), Maheshwari *et al.*, (2016) isolated seven *Candida* species, including *C. albicans* and *C. dubliniensis* and reported *C. albicans* as the most prevalent species.^[44] These results are also relatively consistent with the findings of this study. Studies have shown that, like the results of the present study regarding the simultaneous presence of species, there is a high incidence of mixed infections in people infected with HIV. In a study carried out by Dronda *et al.* on oral lesions of 12 HIV-infected patients, 7 cases of the simultaneous presence of *C. albicans* and *C. krusei*, 4 cases of the simultaneous presence of *C. albicans* and *C. glabrata*, and one case of

the simultaneous presence of *C. albicans* and *C. Tropicalis* were reported.^[45] The differences in the species reported in Dronda *et al.*'s study and those reported in the present study may result from differences in the population under study and time difference because time, as well as medical and pharmaceutical progress, are considered as the key elements in the presence and identification of non-*albicans* species. In addition, it is worth noting that Dronda *et al.*'s study, contrary to the present study, was carried out on individuals with oral lesions, which can be an important reason for differences in species.

The similarity of the results of this study, which was conducted on oral samples of addicts, to the studies conducted on people infected with the HIV virus, can be due to the physiological and immunological similarities of these two groups of people as well as the probability of co-occurrence of AIDS and addiction. Studies have denoted that drugs have a very destructive effect on the immune system and generally there is a direct relationship among the interaction of addiction, ineffectiveness of the immune system, increased pathogen acquisition, creation of different patterns in the development of diseases and chronic infections.^[19,46-49] The HIV and drug use both significantly affect cellular immunity and especially T lymphocytes so that they interfere with the ratio of CD4+/CD8+ cells while decreasing CD4+ cells.^[21,50,51] It seems that geographical climate, time, underlying factors, population and type of the sample under study as well as the method used can probably contribute to the presence of various species of *Candida* in communities and the reason for the differences between the results of the present study and the findings of the inconsistent studies.

Conclusion

This study clearly showed the noticeable presence of *C. albicans* and non-*albicans* species such as resistant *C. glabrata* and *C. dubliniensis* in oral samples of drug

smokers, which could be a warning for the presence of resistant species in the abusers who smoke drugs.

Acknowledgments

We express our thanks to all participants, as well as the staff and authorities of the Addiction Treatment Centers, collaborating with this project. We would also like to express our sincere appreciation to Dr. Hooman Manshaei, the head of the Scientific Community of Addiction Therapists of Isfahan, Iran, for his notable assistance in coordinating for sampling.

Financial support and sponsorship

The following study was performed with a Grant (No. 394770) from Research Deputy of Isfahan University of Medical Sciences.

Conflicts of interest

There are no conflicts of interest.

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