

## Oral candidal colonization in immunocompromised patients

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### ABSTRACT

**Objective:** 1. To identify Candida species isolated from saliva of prediabetically immunocompromised (cancer, diabetic) patients and control group. 2. To test the susceptibility of all the isolated yeasts against 6 antifungal agents, namely amphotericin B, nystatin, fluconazole, ketoconazole, voriconazole and itraconazole. 3. Then to compare the growth and density of yeasts from immunocompromised patients to those from healthy control group.

**Patients and Methods:** This study included 120 saliva samples from immunocompromised patients, 60 of them with cancer and under treatment, the other 60 patients have uncontrolled diabetes mellitus, attending the Al-Wafa'a Center for diabetic patients and the consultative clinic of Oncology and Nuclear Medicine Hospital. The clinical specimens were collected during the period from January-June 2011. In addition to 60 saliva samples from healthy individuals. The identification of the isolated yeasts was carried by fluorescent and/or light microscope, culture on different types of media, biochemical tests and API-20 C system. Susceptibility test to six antifungal agents was prepared for each isolate.

**Results:** Eighty seven (72.5%) patients showed the presence of yeasts in their saliva in comparison to 18(30%) from the control group. Eighty four isolates from genus Candida identified into 8 species mainly C. albicans. The isolates showed the best susceptibility to amphotericin B.

**Conclusions:** A higher frequency of yeasts were detected in the immunocompromised patients in comparison to the control group. This is considered a risk factor for infection. Candida albicans is the main isolate but there was a shift to non –albicans Candida spp. in immunocompromised patients. This means that are more resistant to antifungal agents.

**Keywords:** Candidal colonization, immunocompromised patients.

١. تمييز أنواع الفطريات المأخوذة من اللعاب لمرضى ذوي المناعة المنقوصة ظاهرياً (الأورام، السكري) و من أفراد أصحاء يمثلون مجموعة السيطرة. ٢. إجراء فحوصات الحساسية لكل الخمائر المعزولة باستعمال ٦ أنواع من المضادات الفطرية وهي (amphotericin B, nystatin, fluconazole, ketoconazole, voriconazole and itraconazole). ٣. مقارنة نمو و كثافة الخمائر المعزولة من المرضى ذوي المناعة المنقوصة مع مجموعة السيطرة.

: تضمنت الدراسة ١٢٠ عينة لعاب من مرضى ذوي المناعة المنقوصة، ٦٠ منهم كانوا من مرضى الأورام و تحت العلاج و المرضى ٦٠ الآخرون كانوا يعانون من داء السكر غير المسيطر عليه، وجميع المرضى كانوا من المراجعين للعيادات الاستشارية لمستشفيات ابن سينا و الطب النووي لعلاج الأورام و مركز الوفاء لعلاج مرضى السكري. تم جمع العينات السريرية للفترة من كانون الثاني – حزيران ٢٠١١. كما تضمنت الدراسة مجموعة السيطرة التي شملت ٦٠ عينة لعاب من أفراد أصحاء. تم تمييز الخمائر المعزولة بواسطة الفحص المباشر باستخدام مجهر التآلق و/ أو المجهر الضوئي والزرع على أوساط مختلفة و بإجراء الفحوصات الكيمحيوية واستخدام نظام API-20 C. فحوصات الحساسية لكل الخمائر المعزولة ف يتم إجراؤها باستعمال ٦ أنواع من المضادات الفطرية.

: ظهرت الخمائر في لعاب (٧٢.٥%) ٨٧ مريض بالمقارنة مع مجموعة السيطرة (٣٠%) ١٨ تم تمييز ٨٤ عزلة من جنس المبييضات و صنف الى ثمانية أنواع وخصوصا المبييضات البيضاء. العزلات أظهرت استجابة جيدة للمضاد الفطري amphotericin B .

: النسبة الأعلى من الخمائر ميزت في المرضى ذوي المناعة المنقوصة بالمقارنة مع مجموعة السيطرة و يعتبر هذا عامل خطر لإصابة المرضى ذوي المناعة المنقوصة بها. وكانت المبييضات البيضاء أهم الأنواع المعزولة مع ملاحظة التحول إلى المبييضات غير البيضاء في المرضى ذوي المناعة المنقوصة و الذين كانوا اشد مقاومة للمضادات الفطرية.

**C**andida species is present in oral cavity in about half of the population without causing disease<sup>1</sup>. Asymptomatic carriage may cause a high risk for Candida infection in immunosuppressed patients<sup>2</sup>. Any factors causing microbiota disequilibrium or mucosal lesion, increase colonization by Candida<sup>3</sup>.

The transformation of Candida from a harmless, obscure commensal to a hypervirulent pathogen under the conditions of dysfunctional host defense system is attributed to the expression of virulence determinants, including elaboration of hydrolytic enzymes, secreted aspartyl proteinase and phospholipase<sup>4</sup>. Recently, it has been reported that there is a shift in the dominant causative agent from Candida albicans to non-albicans Candida spp. including C. parapsilosis, C. glabrata, C. tropicalis and others<sup>5</sup>.

Colonization of Candida is more prevalent in diabetic patients<sup>6</sup>. The induced diabetic state was associated with increased susceptibility to infection by Candida species<sup>7</sup>. Furthermore, Candida spp. remain the most common fungal infection in patients with cancer<sup>8</sup>. The chemotherapeutic agents are toxic and target malignant and normal cells mostly of the oral and gastrointestinal mucosa<sup>9</sup>.

Numerous antifungal agents have been developed, and newer agents are on horizon. This is due to the increased resistance of organisms to

the antifungal drugs which leads to the development of newer agents<sup>10</sup>.

### Patients and methods

The studied individuals were 120 predicatively immunocompromised patients including 60 (50%) cancer patients under treatment with chemotherapy and 60 (50%) uncontrolled diabetic patients of > 5 years duration ( their fasting blood sugar more than 200 mg/dl). The males were 54 (45%) and females were 66 (55%) and their age ranged from 1-80 (mean  $\pm$  SD = 51.48  $\pm$  16.42) years.

On the other hand, 60 apparently healthy individuals were included during the study as a control group. These individuals were sex and age matches with the immunocompromised patients.

### The studied samples

A total of 120 saliva samples were collected from the patients in Al-wafa'a center for diabetes and consultative clinic of Oncology and Nuclear Medicine Hospital. Moreover, saliva samples were obtained from 60 normal individuals as a control.

Saliva samples were collected in early morning from patients and control group in dry sterile wide mouth containers. The samples brought to the laboratory without any delay.

### Processing of the clinical samples and isolation of yeasts

A loopful (0.1) of saliva from each sample was inoculated on Sabouraud's agar with chloramphenicol and the same on brain-heart infusion (B.H.I)

blood agar. The plates were incubated at 37°C for 2-3 days, and discarded after 5 days if no growth.

Two smears were prepared from each specimen. The first slide was heat fixed and stained by Gram's staining method. The second wet mounted slide with a drop of 20% KOH solution with glycerin and drop of calcofluor solution, then examined under fluorescent microscope<sup>11</sup>.

For the identification of the isolates, different tests were used including germ tube test, chlamydospore formation test, lactophenol mount, and API-20 C system<sup>12,13</sup>.

### Antibiogram test

Disk diffusion method was used to determine the susceptibility of isolated *Candida* spp. against 6 antifungal agents namely nystatin, amphotericin B, fluconazole, voriconazole, itraconazole and ketoconazole.

A tested yeast suspension was compared to 0.5 McFarland scale, then inoculate onto the surface of Muller–Hinton agar plates. After 2-3 minutes, the antifungal disks were placed firmly on the surface of the inoculated plates. The plates were incubated for 24-48 hour at 37°C.

### Statistical analysis

The test used for statistical analysis.

1. Chi –Square test
2. Z- test
3. Mann-Whitney test
4. t-test

### Results

Among the 120 patients, there were 87(72.5%) who showed the presence of yeasts in their saliva in comparison to 18 (30%) of the 60 control group with a significant difference between them ( $p < 0.001$ ).

From the 87 patients with positive yeast isolates 46 (52.9%) of them were cancer patients, and *Candida* spp.

isolates from 41 (47.1%) of them. On the other hand, from the 41 diabetic patients with positive yeast isolate 40 (46%) of them showed *Candida* spp. in their saliva. Other non- *Candida* yeast isolate were detected from 5 (5.7%) cancer patients and one (1.2%) diabetic patients. Eighteen healthy individuals showed *Candida* spp. in their saliva. Mixed *Candida* spp. were identified from 2 samples from cancer patients and one sample of diabetic patient with no significant in the presence of yeasts in cancer and diabetic patients (Table1).

### Candidal colonization

The mean value of yeast density colony forming unit (CFU) was 10020.1 in the saliva of patients and 772.2 in the saliva of control group, with a significant difference between them ( $P < 0.001$ ).

The diabetic patients who were *Candidal* carriers in their saliva, harbored the yeasts in higher density than the cancer patients. On the other hand, the densities of *Candida* in the saliva of cancer and diabetic patients were higher than the control carriers (Fig 1).

### The isolated yeasts

Different tests were used to identify the yeasts including direct examination of the clinical specimens (Fig 2), then culture, germ tube, chlamydospore formation test, and API- 20 C system.

The 84 isolates of genus *Candida* from cancer and diabetic patients were identified into 8 species representing *Candida albicans* (67/ 84, 79.8%) ; *C. glabrata* (5/84, 5.9%); *C.famata* (3/84, 3.6%); *C. ciferrii* (3/84, 3.6%); *C.parapsilosis* (2/84%, 2.4%); *C.guilliermondii* (1/84, 1.1%); *C. kefyr* (2/84, 2.4%); *C.krusei* (1/84, 1.1%). Furthermore, out of the 90 total yeasts isolate, 6 of them were non- *Candida* species namely *Cryptococcus*

neoformans, *C. laurentii*, *Saccharomyces cerevisiae* and *Rhodotorula rubra* with a significant difference between the species of *C. albicans* and other *Candida* and yeast species ( $P < 0.001$ ), Table 2.

For control group, 2 *Candida* species were identified from the 18 isolates. These were *C. albicans* (17/18, 94.4%) and *C. kefyr* (1/18, 5.6%). Statistically, a significant difference was found between patients and control group in the presence of yeasts in their saliva ( $P \leq 0.001$ ), Table 2

### Susceptibility test

The results of the sensitivity test showed that the most effective antifungal agents against the isolated yeasts from saliva in cancer and diabetic patients were amphotericin B (86.4%, 100%) followed by nystatin (81%, 91.6 %); ketoconazole (71.1%, 88.1%); voriconazole (62.6%, 66.3%); fluconazole (57.9%, 48.4%), least effective antifungal agents was itraconazole (23.4 %, 49 %) respectively. On the other hand, the yeasts from the control group showed 100% sensitivity to amphotericin B and nystatin followed by ketoconazole (91.2%); voriconazole (88.3%); fluconazole (85.3%) and itraconazole (79.4%).

Table 1. The number and percentage of *Candida* species and other yeast species isolated from saliva of patients and control group.

Types of diseases	Patients		<i>Candida</i> isolates		Other yeast isolates	
	No.	%	No.	%	No.	%
Cancer	46	52.9	41*	47.1	5	5.7
Diabetic	41	47.1	40**	46	1	1.2
Total	87	100	81	93.1	6	6.9
Control	18	100	18	100	-	-

\* 2 Mixed *Candida* species , \*\* 1 Mixed *Candida* species

Note: There is no significant difference in the presence of yeasts in cancer and diabetic at ( $p < 0.281$ ) by Chi-square test.

Table 2. Distribution of Candida species and other yeasts species in studied subject.

Isolates	Total isolates		Patients				Control group	
			Cancer		Diabetic			
	No.	%	No.	%	No.	%	No.	%
<i>C. albicans</i>	67	74.4	33	36.7	34	37.8	17	94.4
<i>C. glabrata</i>	5	5.6	2	2.2	3	3.4	-	-
<i>C. famata</i>	3	3.3	1	1.1	2	2.2	-	-
<i>C. cifferrii</i>	3	3.3	3	3.4	-	-	-	-
<i>C.parapsilosis</i>	2	2.3	2	2.2	-	-	-	-
<i>C.kefyr</i>	2	2.3	-	-	2	2.2	1	5.6
<i>C. guilliermondii</i>	1	1.1	1	1.1	-	-	-	-
<i>C.krusei</i>	1	1.1	1	1.1	-	-	-	-
<i>C .neoformans</i>	1	1.1	1	1.1	-	-	-	-
<i>C. laurentii</i>	3	3.3	2	2.2	1	1.1	-	-
<i>S .cerevciae</i>	1	1.1	1	1.1	-	-	-	-
<i>R. rubra</i>	1	1.1	1	1.1	-	-	-	-
<b>Total</b>	90	100	48	53.3	42	46.7	18	100

Note : There is a significant difference between the presence of *Candida albicans* and other yeast specie at  $p < 0.001$  by Z-test.

There is a significant difference between patients and control in presence of yeast species in their

clinical specimen at  $p < 0.001$  by Mann-Whitney test.

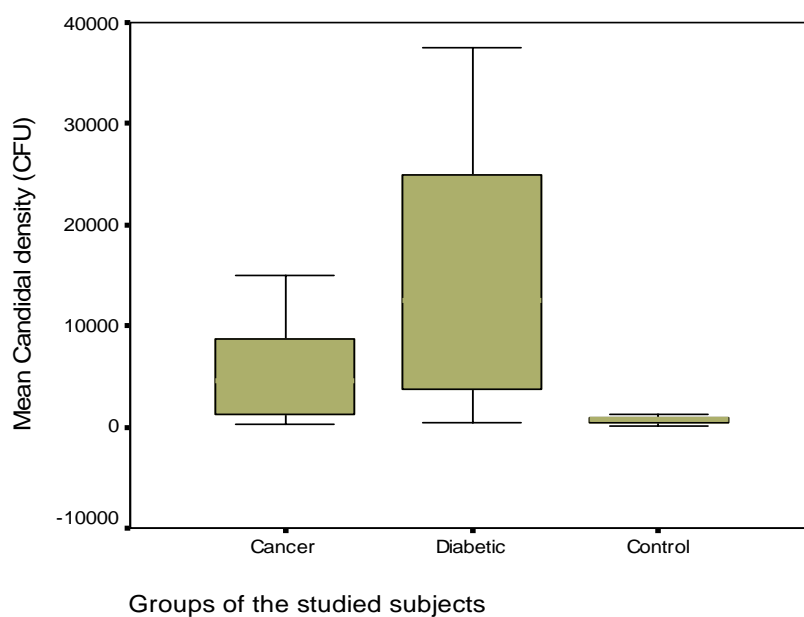


Figure 1. Candidal density in saliva among the studied subjects.

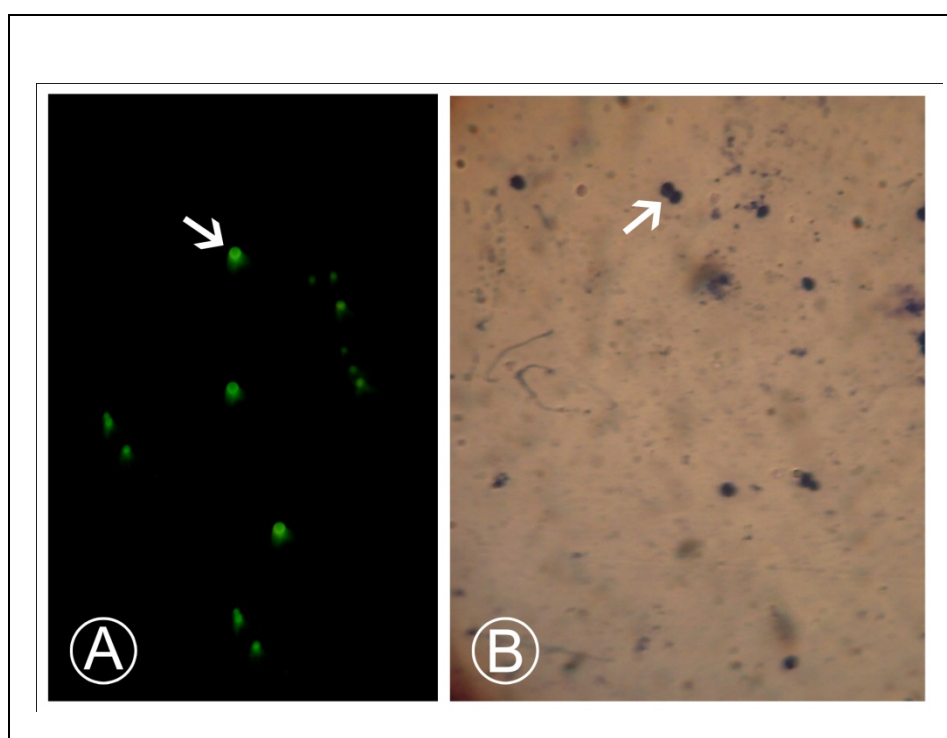


Figure 2. Direct examination of the clinical specimens (saliva) showing budding yeast cell in:

A- 20% KOH mounting with calcofluor stain under fluorescent microscope 40X lens (arrowed).

B- Gram's staining smear under 100X lens (arrowed).

## Discussion

*Candida* establish a biological link with the host which guarantees their saprophytic condition by establishing an ecological equilibrium denoted "amphibionts". When this equilibrium is broken due to different endogenous and/or exogenous factors, these amphibionts behave as opportunistic microorganisms and can invade tissues and cause oral candidiasis or life-threatening disease in patients whose immune defenses have been altered by different factors<sup>14</sup>.

Eighty seven (72.5%) cancer and diabetic patients revealed budding yeast cells in their saliva in comparison to 18 (30%) healthy control. Lotfi-Kamran and Coworkers (2009); Afraseyabi and Coworkers (2011)<sup>15</sup> reported that a higher colonization of *Candida* species was seen in diabetic and cancer patients compared to the control group. Furthermore, the carriage rate for *Candida* is hard to give since it depends on the age and health of the population studied<sup>16</sup>, but a range from 17%-75% has been reported<sup>17</sup>.

From the 52.9 % cancer patients and 47.1% diabetic patients, *Candida* species were identified in 93.1 % of the total isolated yeasts, while non-*Candida* species or other yeasts formed 6.9 %. These results were confirmed by Al-Attas and Amro (2010) as they indicated that the most isolated yeasts were *Candida* species (93.3 %), and the other yeasts were (6.7%). Over the past decades, numerous surveys have documented an increase rate of fungal infection among both oncology and non-oncology patients and the most common isolates were *Candida* species<sup>19</sup>.

## The isolated yeasts and their densities from cancer and diabetic patients

The 90 yeast isolates that were identified during the study included 48 isolates from cancer patients and 42 from diabetic ones with no significant difference between the two groups by using Chi-square test ( $p \square 0.281$ ). This is due to the immunosuppressive status of the patients of the two groups as mentioned by other investigator who stated that Candidal colonization and infection occur in close association with chemotherapy for cancer, immune dysfunction, and malnutrition<sup>20</sup>.

A higher frequency of yeasts were detected in the saliva of both cancer and diabetic patients (53.3 % ; 46.7% respectively) table 2. Other study reported that the frequency of yeasts in saliva of cancer patients were 31.6%, while from diabetic patients were 75.7 %<sup>15,21</sup>. The hyposalivation and xerostomia are considered a common complaint by diabetic and cancer patients especially after chemotherapy, which predispose the oral mucosa to opportunistic infection by *Candida* species<sup>9</sup>.

All the diabetic patients that were studied had a level of glucose in their blood higher than 200 mg /dl, (HbA1C > 9.2). This is confirmed by other investigators who reported that the predisposition of diabetic patients to *Candida* overgrowth has been explained in terms of the enhancement of yeast growth by elevated glucose level<sup>22</sup>.

A significant difference was recorded during the current study between the density ( CFU/ml) of the yeasts in saliva (mean =10020.1) of the patients, and saliva (mean = 772.2) of the control group by t-test ( $p \square 0.001$ ). These results showed that the carriage rate and density of the yeasts was influenced significantly by

systemic factors such as the disease types of the studied patients. Other investigators reported the risk factors for Candidal infection in cancer and diabetic patients are complex<sup>18</sup>. On the other hand, the higher mean of Candidal density was detected in the saliva of the studied diabetic patients (14518.3 CFU/ml) in comparison to cancer patients (6010.9 CFU/ml). This may be due to the long duration of diabetes mellitus patients as recorded in the request forms during the study (Fig 1).

### **Candida species**

Out of the 84 isolates of Candida species, 74.4% were *C.albicans* which showed a significant difference in their presence in comparison to the other Candida and non-Candida yeast species. These results were confirmed by other investigators who stated that *C.albicans* alone outnumbered other species and accounted for 73.7%<sup>15</sup>. Meruman and Coworkers (2007) reported that the most frequent encountered species are *C.albicans*, and non-Candida *albicans* species are isolated in increasing number in medically compromised patients. In the control group, Candida *albicans* also appeared as the main isolate (94.4%) from the 18 healthy individuals. This keeping with other reports that the most common species isolated from the oral cavity of both healthy and diseased cases is *C.albicans*.

### **Antifungal susceptibility test**

Antifungal susceptibility tests are now required to optimize antifungal treatment which gives the emergence of antifungal resistance in some Candida species<sup>24</sup>.

From the data outlined in the results, it was evident that amphotericin B constituted the first choice drug for the treatment of yeasts infection caused by Candida and non-

Candida yeasts species. These results were in agreement with Kabli(2008)<sup>25</sup> and Bernal-Martinez et al., (2010)<sup>26</sup> that reported amphotricin B in vitro was the most active drug against yeast species. The others most effective antifungal drugs were nystatin, ketoconazole and voriconazole. The least effective antifungal agents against *C. albicans* were fluconazole and itraconazole. On the other hand, the isolated Candida species from control group showed good sensitivity to all the 6 antifungal agents. Fluconazole has been widely used for the prophylaxis and the treatment of different types of candidiasis because its low toxicity and the ease of administration, but it can be associated with a shift of species and resistance pathogens<sup>27</sup>. Magaldi and Coworkers (2001)<sup>28</sup> found that 9.8% and 4.9% of *C. albicans* isolated from patients with no previous antifungal treatment were resistant to fluconazole and itraconazole respectively, which increased to 44.7% and 44.15% after treatment with these drugs.

In conclusion: the direct yeast identification procedures and culture, in addition to biochemical tests, identify different yeasts in 72.5% (87/120) of the immunocompromised patients from their saliva. Eight Candida species and 4 non-Candida species have been identified. The apparently healthy control group showed 2 Candida species isolates in 30%(18/60) of them. The antifungal susceptibility test was done for the first time and showed a good response to amphotericin B but increasing resistance to fluconazole and have been controlled. The increasing growth and density of yeasts in the immunocompromised patients in comparison to the control group, is considered a risk factor for the infection by these species, and has be



to controlled by effective antifungal agents.

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