Unusual yeasts isolated from immunocompromised patients

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ABSTRACT

Objectives: To identify rare fungi from saliva and urine of immunocompromised patients, and apparently healthy controls and to test the susceptibility of the rare fungi to antifungal agents.

Patients and methods: One hundred and twenty immunocompromised (cancer, diabetic) patients were enrolled in this study. The clinical specimens were collected from January-July 2011 and included saliva (110) and urine (10) samples, in addition to saliva (6) and urine (6) samples from a control group. The identification process employed direct examination, culture, biochemical tests and API-20 C system test. Susceptibility test to six antifungal agents was prepared for each isolate.

Results: Among 110 yeast isolates, only 7 (6.3%) were categorized as unusual yeasts belonging to three genera. Four isolates were Cryptococcus laurantii, one C.neoformans, one Saccharomyces cerevisiae, and one isolate was Rhodotorula rubra. All the isolates have various susceptibility to antifungal agents.

Conclusion: Many opportunistic fungi are important uncommon pathogens in saliva and urine of immunocompromised patients.

The medical advance has improved preventive, diagnostic and therapeutic capabilities for a variety of diseases. However, certain therapies like the cytotoxic and others that lead to immunosuppression may predispose the host to an expanding group of opportunistic pathogens. The increasing population of immunocompromised patients has led
to infections with less common organisms. On the other hand, the severely immunosuppressed patients have a state in which the immune system is suppressed by medications during the treatment of cancer or other disorders as chronic renal failure and diabetes.

Most fungal infections are caused by common opportunistic fungi as *Candida* species and *Aspergillus* spp. A number of non-*Candida* yeast like fungi found an opportunity to colonize and infect immunocompromised patients. These organisms may occupy environmental niches or be found in food and water and can be normal human microbial flora. The list of these opportunistic yeasts is long, but the main pathogen is *Cryptococcus* spp., in addition to *Rhodotorula* spp., *Malassezia* spp., and *Saccharomyces* spp., which emerging as significant causes of infection in immunocompromised patients.

The techniques of antifungal susceptibility test have now been standardized, and *in vitro* antifungal susceptibility test results of the drug tested can be used to predict *in vivo* clinical response. The recognition of unusual yeasts as agents of sometime life-threatening infections and their unpredictable antifungal susceptibility increase the burden on the clinical microbiology laboratory to complete species identification and determine minimal inhibitory (MIC) concentration. The aim of the study is to detect rare fungi from saliva and urine of immunocompromised (cancer and diabetic) patients, and to test their susceptibility to antifungal agents.

### Materials and methods

One hundred and twenty immunocompromised patients were included in this study. The males were 60 (55%) and females were 64 (50%). The age of the patients ranged from 1-80 (mean±SD: 53.4±17.43) years. The immunocompromised patients were: 56 (50%) diabetic patients with uncontrolled diabetes mellitus of >5 years duration, and their fasting blood sugar more than 200 mg/dL, and 60 (50%) patients with different types of cancer, and under treatment with chemotherapy.

Sixty apparently healthy individuals were included in the present study as a control group. They were 72 (55%) males and 33 (55%) females. Their ages ranged between 1-80 (mean±SD: 55.21±9.63) years. These individuals were sex and age matches with the immunocompromised patients.

A total of 84 samples were collected from patients in Alwafa'a Center for diabetes, Center of Outpatients of Oncology and Nuclear Medicine Hospital, and Center of Outpatients in Ibn-Sina Teaching Hospital. The samples were consisted of 42 saliva and 42 urine samples. From the 60 control individuals, both saliva and urine samples were also collected and processed in the same manner as for the patients.

Early morning saliva samples were collected from all patients in dry sterile wide mouth containers. The samples brought to the laboratory within one hour after collection. The mid stream urine in the early morning was collected from each patient after cleaning the genital area into dry sterile
plastic wide mouth container. The specimens were transferred to the laboratory without any delay.

The saliva samples were used directly for culture and direct microscopical examination. Urine samples were centrifuged for 3 minutes at 1600 rpm. The supernatant was discarded, and the sediment was used for culture and direct microscopical examination.

**Isolation of the yeasts**

A loopful (0.1 ml) of the clinical specimen (saliva and urine) was inoculated onto each of Sabouraud's agar and Brain–Heart Infusion (BHI) blood agar. The specimens were streaked on all the surfaces of the media to obtain separated colonies. The plates then incubated aerobically at 37ºC for 2-3 days. The cultures were examined after the second day of incubation and considered negative after the third day, then discarded after five days if no growth was obtained.

**Direct examination**

Two smears were prepared from each clinical specimen. Wet mounted slide with a drop of calcoflour solution and a drop of 2% KOH solution with glycerin, then examined under 40X fluorescent microscope. The second heat fixed smear was stained by Gram's method and examined under oil immersion lens.

**Identification of the isolates**

Lactophenol mount, biochemical tests (API-20), urease, germ tube test, morphology on cornmeal agar Tween 80, and capsule stain were used for identification of the yeast species.

**Antifungal susceptibility test**

The standard disk diffusion method was used to determine the sensitivity of the uncommon yeast against different antifungal agents (nystatin 50 µg, amphotericin B 2 µg, fluconazole 50 µg, voriconazole 1 µg, itraconazole 1 µg and Ketoconazole 51 µg) according to Vale-Silva and Buchta.

A suspension of the tested yeast compared to 0.5 McFarland scale was prepared in a test tube. A sterile cotton swab soaked in yeast suspension was used to inoculate the organism onto the surface of Muller–Hinton agar plates. The plates were left at room temperature for several minutes in order to dry, then the antifungal disks were placed firmly on the surface of the inoculated plates using a sterile forceps. The plates were incubated for 48-72 hours at 37ºC.

**Results**

Three genera of unusual yeasts were identified in seven (6.3%) out of 111 yeast isolates from 111 cancer and diabetic patients from both saliva and urine. No isolates of the opportunistic unusual yeasts were identified from the control group (Table 1).

Five isolates of genus Cryptococcus were detected from saliva and urine of the patients. One isolate from saliva of cancer patient identified as *C. neoformans*. The other 4 isolates were identified as *C. laurantii*, 7 of them detected in saliva of cancer patients, one from urine of the same group of patients, While the fifth isolate from saliva of diabetic patient (Table 2). Different tests were used to identify the 5 isolates. These were direct examination of the clinical specimens with different stains.
including capsule stain, and culture on two types of media (Fig ١-A,B). Additional identification tests namely urease test and API-٦•٥•٩ system which identify the species of each isolate.

One isolate of the genus *Saccharomyces* was detected from the saliva of cancer patient (Table ١). This isolate grew well on Sabouraud’s agar with dry white colonies and microscopically showed budding yeast cells with fragments of mycelial elements (Fig ١-C,D), and then identified as *S. cereviciae* by API-٦•٩ C system.

*Rhodotorula* was detected in saliva of one cancer patient. Showing pink mucoid colony on Sabouraud’s agar and large budding yeast cells in lactophenol mount (Fig ١-E,F). The isolate gave positive urease test and identified as *R. rubra (mucilaginosa)* by API-٦•٩ C system.

### Sensitivity test

The results of the sensitivity tests with the six antifungal agents used are shown in Table ٩. The one isolate of *C.neoformans* from saliva of cancer patient showed sensitivity to nystatin, fluconazole and itraconazole only. On the other hand, the ٩ isolates of *C. laurantii* from saliva of the patients were sensitive to ketoconazole and resist to itraconazole. Two isolates sensitive to Polyenes and voriconazole, and one sensitive to fluconazole. The one isolate of *C. laurantii* obtained from urine was sensitive only to amphotericin B and ketoconazole, and the sensitivity to nystatin was excluded. *Saccharomyces cereviciae* showed sensitivity to polyenes andazole compound except itraconazole. Lastly, *R.rubra* was sensitive only to ketoconazole and voriconazole.

#### Table ١. Number and percentage of *Candida* species and unusual yeasts isolated from the studied subjects.

<table>
<thead>
<tr>
<th>Studied subjects</th>
<th>Total isolates</th>
<th>Candida species</th>
<th>Unusual yeast species</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>Cancer patients</td>
<td>٥٦</td>
<td>٥٠.٩</td>
<td>٥٠</td>
</tr>
<tr>
<td>Diabetic patients</td>
<td>٥٤</td>
<td>٤٩.١</td>
<td>٥٣</td>
</tr>
<tr>
<td>Total</td>
<td>١١٠</td>
<td>١٠٠</td>
<td>١٠٣</td>
</tr>
<tr>
<td>Control group</td>
<td>١٨</td>
<td>١٠٠</td>
<td>١٨</td>
</tr>
</tbody>
</table>

Number of specimens with ٩ species = ٣
Isolates from saliva and urine at the same time obtained from ٩٧ patients.
Table 2. Number and percentage of unusual yeast species isolated from saliva and urine of the immunocompromised (cancer, diabetic) patients.

<table>
<thead>
<tr>
<th>Isolate species</th>
<th>Total isolates</th>
<th>Cancer patients</th>
<th>Diabetic patients</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>saliva</td>
<td>urine</td>
<td>saliva</td>
<td>urine</td>
<td></td>
</tr>
<tr>
<td>No. %</td>
<td>No. %</td>
<td>No. %</td>
<td>No. %</td>
<td>No. %</td>
<td>No. %</td>
<td>No. %</td>
</tr>
<tr>
<td><em>Cryptococcus neoformans</em></td>
<td>1</td>
<td>14.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Cryptococcus laurantii</em></td>
<td>4</td>
<td>57.1</td>
<td>2</td>
<td>28.3</td>
<td>1</td>
<td>14.3</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>1</td>
<td>14.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Rhodotorula rubra</em></td>
<td>1</td>
<td>14.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>7</td>
<td>100</td>
<td>1</td>
<td>14.3</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3. Number of sensitive and resistant unusual yeasts isolated from saliva and urine of patients to the antifungal agents.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Polyenes group</th>
<th>Azoles compound</th>
<th></th>
<th></th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Nystatin</td>
<td>Amphotericin-B</td>
<td>Fluconazole</td>
<td>Ketoconazole</td>
<td>Voriconazole</td>
<td>Itaconazole</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td><em>Cryptococcus neoformans</em></td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>Cryptococcus laurantii</em></td>
<td>4</td>
<td>*</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>Rhodotorula rubra</em></td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>7</td>
<td>5</td>
<td>2</td>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

S= Sensitive; R=Resistant

*One isolate of *Cryptococcus laurantii* from cancer patients in urine showed sensitivity to amphotericin B and ketoconazole. Sensitivity to nystatin was excluded for this species.
Fig. 1 unusual yeasts:
A- *Cryptococcus neoformans* on B.H.I blood agar showing yellowish brown mucoid colonies after 9 days of incubation at 37°C.
B-Capsule stain for *C. neoformans* from culture showing the capsules around the budding yeast cells (arrowed).
C-*Saccharomyces cerevisiae* on Sabouraud's agar showing white, dry colonies after 7 days of incubation at 37°C.
D-Lactophenol mount of *S.cerevisiae* showing budding yeast cells with short pseudohyphae (arrowed).
E-*Rhodotorula rubra* on Sabouraud's agar showing pink mucoid colonies after 7 day of incubation at 37°C.
F-Lactophenol mount of *R. rubra* showing large budding yeast cells (arrowed).

**Discussion**
The opportunistic unusual yeasts that are sometime found on the skin, in urine, sputum, and in the digestive tract of healthy individuals may be responsible for deep infections, as well as for cutaneous mucosal diseases. Such deep infections are facilitated by immunosuppression, or by factors that enable these organisms to proliferate in different sites of the body and to penetrate deep in tissues.

The identification of the isolated yeasts in the present study depended on the colonial morphology, microscopic examination and biochemical tests. Winn and Coworkers (18) mentioned that most fungal identification are based primarily on the assessment of colony morphology and microscopic features, but key biochemical tests may be required to differentiate between genera and species within a given group.

**The isolates**
*Cryptococcus*: two species in this genus were identified. *Cryptococcus neoformans* was isolated from saliva of cancer patient. Karkowska-Kuleta and
Coworkers (١٩٠٢) reported that C. neoformans is less known than Candida spp., but now a day the morbidity and mortality caused by cryptococcosis is a significant problem. Other investigators reported that C. neoformans is a fungus that cause disease in people with immunodeficiencies associated with cancer related chemotherapy١٩٠٢. This species was isolated and identified previously in our locality by the conventional method١٩٠٢٢ and API-١٩٠٢٤ C system١٩٠٢٥. The second species was C. laurantii which isolated from ٢٠٢٢٢ clinical specimens, and identified by API-١٩٠٢٦ C system for the first time. It should be noted that in a recent study comparing efficacy of detection by API-١٩٠٢٧ C system in clinical laboratory, seven of seven C. laurantii isolates were correctly identified by using the API-١٩٠٢٨ C system١٩٠٢٩. Moreover, identification of Cryptococcal organisms based on solely upon phenological characteristics may result in mis-identification of Cryptococcal species. Different authors mentioned that C. laurantii is only rarely isolated clinically, but it has recently been implicated in many cases among immunocompromised individuals١٩٠٢١, and as a non-neoformans Cryptococcal spp. may be considered an emerging infective species١٩٠٢٢.

Saccharomyces:. One isolate of S. cerevicae was detected during the study from saliva of cancer patient. Saccharomyces spp. are now among emerging causative agents of opportunistic mycoses in patients who are immunocompromised due to various reasons١٩٠٢٣. Enache-Angoulvant and Hennequin (١٩٠٢٥) mentioned that Saccharomyces organisms are increasingly reported as agents of invasive infection especially in immunosuppressed or critically ill patients, and the rate of carriage varies according to the population investigated.

Rhodotorula: This genus contain several spp. that may be present on the skin and in the sputum, urine and feces, and have been implicated as an infrequent cause of infection١٩٠٢٤. The main spp. is R. rubra١٩٠٢٥. One isolate of R. rubra from saliva of cancer patient was identified in the present study. It was reported that the risk factor for infection include underlying immunosuppression١٩٠٢٦. Other investigators mentioned that most infections caused by Rhodotorula spp. have been associated with patients who have solid tumor, diabetes, endocarditis and AIDS١٩٠٢٧.

Susceptibility to antifungal agents

The antibiogram of the unusual yeasts range from resistant to the most recentazole and amphotericin B to those are highly susceptible to all antifungal agents١٩٠٢٨. The in vitro susceptibility profile of isolates of uncommon yeast that were detected in the clinical specimens was studied against١٩٠٢٩ antifungal agents. The susceptibility of the isolates within genus Cryptococcus was varies. Cryptococcus neoformans showed susceptibility to nystatin, fluconazole and ketoconazole. The١٩٠٢٠ isolates of C. laurantii varied in their sensitivity, ٢٠٢٢١ of them were susceptible to amphotericin B, while ١٩٠٢٢ two to voriconazole and one to fluconazole. Moreover, all the isolate were resistant to itraconazole but sensitive to ketoconazole. The one isolate from urine was excluded from the test of sensitivity to nystatin. Averbuch et al.١٩٠٢٣ found that non –neoformans Cryptococcus spp. are susceptible to amphotericin B and various azole. However, some isolates of C. laurantii were found to be resistant to fluconazole. Moreover, Bernal-Mortinez et al.١٩٠٢٤ reported that amphotericin B was in vitro the most active compound against all non-
Cryptococcus neoformans spp., fluconazole exhibit a limited activity, particularly against *C. laurantii*, while voriconazole and Itraconazole were active against most isolates but a significant rates of decreased susceptibility was noted.

The one isolate of *R. rubra* showed susceptibility to ketoconazole and voriconazole only. The wide spread antifungal prophylaxis with triazole antifungal agents mainly fluconazole in immunocompromised patients may allow the emergence of more resistant yeasts such as *Rhodotorula* spp.

*Saccharomyces cerevisiae* which detected from one clinical specimen showed sensitivity to all the antifungal agents except itraconazole. Different reports mentioned that the majority of serious infections due to *Saccharomyces* have been treated with amphotericin B. Moreover, ketoconazole, fluconazole and voriconazole exhibit good efficacy against *S. cerevisiae*. In conclusion, unusual fungi are important cause of opportunistic infections in immunocompromised patients. All the isolated fungi were sensitive to ketoconazole but resistant to itraconazole with various sensitivity to other antifungal agents.

References

13. Vartivarian SE, Anaissie EJ, Bodey JP. Emerging pathogens in immunocompromised patients:


