

Opportunistic fungi in lower respiratory tract infection among immunocompromised and immunocompetent patients

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ABSTRACT

Objectives: (1) to identify the opportunistic fungi from sputum and bronchial wash of patients with lower respiratory tract (LRT) infections in immunocompromised (IC) and immunocompetent (IP) patients, and apparently healthy controls, (2) to detect antibodies against *Aspergillus* species by double immunodiffusion test (ID).

Subjects and methods: Three hundred patients suffering from LRT infections of both IC (150/300) and IP (150/300) patients were included in the study. The clinical specimens collected were samples of sputum (247), bronchial wash (80), and blood (300). The control group was 50 apparently healthy individuals, from whom sputum and blood were obtained. The identification of the isolated fungi was carried out by direct fluorescent and/or light microscopy, culture on different media, and biochemical tests. Moreover, the serums of patients with *Aspergillus* isolates were tested by double ID test for the detection of specific antibody.

Results: One hundred eighty patients showed fungal elements in their clinical specimens (60%). Two hundred four funguses were detected, including 24 samples with 2 types of isolates. The identified fungi were encountered from both IC (60.9%) and IP (39.1%) patients with a significant difference between them ($p < 0.001$). Nine opportunistic genus-species were identified. Five were filamentous type namely *Aspergillus* spp., *Penicillium* spp., *Cladosporium* spp., *Fusarium* spp., and *Geotrichum* spp., while the other 4 were unicellular organisms including *Candida* spp., *Saccharomyces cerevisiae*, *Cryptococcus neoformans*, and *Rhodotorula rubra*. In the control group, 36% showed fungal isolates in their sputa, and the ID test showed a positive result for antibody in only one patient with *Aspergillus* isolate.

Conclusions: Many opportunistic fungi are important uncommon pathogens in LRT infections in IC patients. The ID test is of limited value for the detection of specific antibody of *Aspergillus* spp.

Keywords: Opportunistic fungi, fungi in L.R.T.

الخلاصة

الهدف: (١) تمييز الفطريات المأخوذة من القشع والغسيل القصي لمرضى ذوي المناعة المنقوصة والمناعة الكفوءة للمصابين بجمع الجهاز التنفسي السفلي ومن أفراد أصحاء يمثلون مجموعة السيطرة. (٢) الكشف عن الأجسام المضادة لفطر *Aspergillus* بواسطة فحص الانتشار المناعي المزدوج.

عينات الدراسة وطرائق العمل: تضمنت هذه الدراسة ٣٠٠ مريضاً يعانون من جمع الجهاز التنفسي السفلي ٣٠٠/١٥٠ من ذوي المناعة المنقوصة و ٣٠٠/١٥٠ من ذوي المناعة الكفوءة. ان العينات السريرية التي تم جمعها كانت ٢٤٧ عينة من القشع، ٨٠ عينة من الغسيل القصي، و ٣٠٠ عينة من الدم. كما تضمنت الدراسة مجموعة السيطرة التي شملت ٥٠ من الأشخاص الأصحاء وتم الحصول منهم على ٥٠ عينة من القشع و ٥٠ عينة من الدم. تم تمييز الفطريات المعزولة بواسطة الفحص المباشر باستخدام مجهر التآلق الضوئي و/أو المجهر الضوئي، والزرع على أوساط مختلفة، وكذلك الفحوصات

الكيموحيوية. بالإضافة إلى ذلك، تم إجراء فحوصات على مصل دم المرضى الذين عزلت منهم الـ *Aspergillus spp.* باستخدام فحص الانتشار المناعي المزدوج للكشف عن المضاد الخاص. **النتائج:** أظهر ١٨٠ مريضاً وجود عناصر فطرية انتهازية في عيناتهم السريرية (٦٠%). تم تشخيص ٢٠٤ فطر، وقد وجد نوعين من الفطريات في ٢٤ عينة. تم حساب الفطريات المعزولة من المرضى ذوي المناعة المنقوصة (٦٠,٩%) ومن ذوي المناعة الكفوءة (٣٩,١%) وكان هناك فرق معنوي بينهما ($p < 0.001$). ميّزت ٩ أجناس وأنواع من الفطريات الانتهازية. كان ٥ منها من النوع الخيطي وهي *Penicillium, Aspergillus, Candida spp.* و *Geotrichum, Fusarium, Cladosporium, Rhodotorula rubra, Cryptococcus neoformans, Saccharomyces cereviciae,* أظهر ٣٦% من مجموعة السيطرة وجود عزلات فطرية في القشع وأظهر فحص الانتشار المناعي المزدوج نتيجة إيجابية لوجود الأجسام المضادة في مريض واحد فقط عزل منه الـ *Aspergillus*. **الاستنتاجات:** تعد بعض من الفطريات الانتهازية من الممرضات المهمة غير الشائعة في أخماج الجهاز التنفسي السفلي عند المرضى ذوي المناعة المنقوصة. إن فحص الانتشار المناعي المزدوج ذو قيمة محددة للكشف عن الأجسام المضادة.

Many opportunistic fungi as *Candida* species, *Aspergillus* species, *Cryptococcus neoformans* and others tend to cause pneumonia in patients with congenital or acquired defects in their host defenses ⁽¹⁾. On the other hand, opportunistic mycoses are infections due to fungi with low inherent virulence, which mean that the pathogens constitute limitless number of fungi that are present as saprophytes or endogenous as commensals ⁽²⁾.

Aspergillus is a filamentous cosmopolitan fungus found in nature. Its pathogenicity is low and primary aspergillosis rarely occurs in man ⁽³⁾. Aspergillosis is one of the earliest fungal diseases recognized and the major portal of entry for infection is the respiratory tract. Nosocomial infection may be associated with dust exposure during building renovation or construction. The most important nosocomial infection due to the *Aspergillus* species is pneumonia ⁽⁴⁾.

Hyalohyphomycetes are unusual hyaline fungal pathogens cause hyalohyphomycosis, where the tissue morphology of the causative organism is mycelial. The etiological agents include species of *Penicillium, Paecilomyces, Acremonium, Fusarium,* and *Scopulariopsis* ⁽⁵⁾.

Phaeohyphomycetes are fungi with dark-walled septated hyphae and sometimes yeast or a combination of both forms in tissue that cause phaeohyphomycosis. These fungi include various dematiaceous hyphomycetes

specially species of *Exophiala, Phialophora, Bipolaris, Cladosporium* and *Alternaria* ⁽⁶⁾.

Candida are small yeasts that reproduce by budding. There are more than 150 species of *Candida*, about 10 of them cause diseases in human ⁽⁶⁾. The main species is *Candida albicans*, and the others are *C. tropicalis, C. krusei, C. parapsilosis* ⁽⁷⁾. *Candida* species are normal commensals of human. They produce a wide variety of infections, and distinguishing between colonization and infection can sometime be a challenge ⁽⁸⁾. They are the most common cause of opportunistic mycoses worldwide. *Candida* pneumonia is one of the most challenging of all the *candida* infection.

Cryptococcus have 37 species, the main human pathogen is *C. neoformans*. The organism exists as a yeast in both nature and tissue. It is 4-6 μm in diameter, with a capsule. Pulmonary infection with *C. neoformans* may have several untoward clinical sequelae ⁽⁹⁾. Most of them are viewed as being "clinically silent" ⁽¹⁰⁾, but symptomatic disease can occur. Pulmonary cryptococcosis is rare in the immunocompetent individuals ⁽¹¹⁾, or may be asymptomatic and resolve spontaneously. The disease commonly occurs in AIDS. It occurs also in those, who on cytotoxic chemotherapy, those receiving corticosteroids or patients with hematological malignancy ⁽¹²⁾.

A group of rare yeasts, which are normally non pathogenic or with low virulence, and considered as occasional part of normal flora

in sputum, urine and stool⁽⁶⁾. The main genera of these yeasts are *Trichosporon*, *Rhodotorulla*, and *Saccharomyces*. The infections caused are opportunistic and occur in patients with altered host defense⁽¹³⁾.

Hence, the main aim of the present study is to identify the opportunistic fungi isolated from immunocompromised and immunocompetent patients with LRT infections.

Subjects and methods

Patients: Three hundred patients with lower respiratory tract (LRT) infection were included in this study that extended from April 2007 to June 2008. The males were 175 (58.3%) and females were 125 (41.7%). The age of the patients ranged from 1-89 (mean \pm SD = 55.44 \pm 17.9) years. The patients were either immunocompromised (IC) or immunocompetent (IP) with equal number, 150 (50%) for each group.

The immunocompromised patients had the following primary or underlying diseases:

1. Different types of carcinoma and leukemia: 69/150 (46%).
2. Uncontrolled diabetes mellitus of >5 years duration: 38/150 (25.3%).
3. Old tuberculous patients (Negative AFB at the time of the study: 16/150 (10.7%).
4. Chronic diseases under long – term corticosteroids therapy: 27/150 (18%).

Normal control: Fifty apparently healthy individuals were enrolled in the current study as a control group. They were 28 (56%) males and 22 (44%) females. Their ages ranged between 15-60 (mean \pm SD = 36.1 \pm 12.3) years. These individuals were 20 hospital workers, 10 medical staff, 10 patients' companions, and 10 individuals from the general population visiting the hospitals.

Studied samples: A total of 627 samples were collected from patients in Teaching Hospitals (RCU, Bronchoscopy Unit and Wards). The samples consisted of 247 sputum and 80 bronchial wash (27 patients with both sputum and bronchial wash at the same time). Three hundred samples of blood obtained from all patients. From the 50 control individuals, both sputum and blood samples

were also obtained and processed in the same manner as for patients.

The sputum of each patient was shaken, by a vortex for 3-5 minutes for homogenization. The B. wash was centrifuged for 5 minutes, then the sediment was used for culture and direct examination. The blood was centrifuged for 3 minutes then the serum stored at -20°C until use.

Isolation of the fungi

The clinical specimens (B. wash and/or sputum) of the patients and sputum of control individuals were inoculated onto brain heart infusion (BHI) blood agar and double plates of modified Sabouraud's agar with antibiotics then incubated at 28-30°C for several days⁽¹⁴⁾. The cultures were examined daily after the third day of incubation, if no growth was obtained after 2 weeks, it was considered negative and discarded.

Direct examination

Four slides were prepared from each clinical specimen. Two wet mounted slides, one with 20% KOH solution and the second with 20% KOH and calcofluor solution, then examined under 40X of light and fluorescent microscopes respectively⁽¹⁴⁾. The third heat fixed smear was stained by Gram's method and examined under oil immersion lens, and the last slide was nigrosine stained smear.

Identification of the isolates

Biochemical tests (API-C, urease), germ tube test, morphology on cornmeal agar Tween 80, slide culture technique and growth and characteristics on Czapek's agar were used for identification of yeasts and molds.

Double immunodiffusion technique (Ouchterlony) was used to test the presence of antibody in serum of patients with *Aspergillus* isolates against antigen⁽¹⁵⁾. The *Aspergillus* antigens and anti-*Aspergillus* antibody used were from Meridian, Bioscience, Inc. Cincinnati, Ohio with Cat. No. 100501 and 100901 respectively.

The data were analyzed statistically as follows:

1. Standard statistical methods were used to describe the results of the study: mean,

standard deviation, number and percentage.

2. Observed / expected χ^2 square was used to find the differences between the percentages.

The statistical results considered significant at $p < 0.05$.

Results

Among the 300 cases studied, a total of 204 opportunistic fungi were detected from IC (124) and IP (80) patients (Table 1). There was a significant difference ($P < 0.001$) between the number of fungal isolates encountered in IC and IP patients.

Out of the 50 control individuals, 18 (36%) showed positive results for opportunistic unicellular fungi only. The isolates obtained from their sputum were 14 (28%) *C. albicans*, 2 (4%) *S. cerevisiae*, and 2 (4%) unidentified *Candida* species.

Opportunistic (monomorphic) molds

Aspergillus

Four species of the genus *Aspergillus* were detected from the clinical specimens. The total isolates were 12, five of them were *A. flavus*, 3 *A. fumigatus*, 3 *A. niger*, and 1 *A. terreus*. The number and percentage of the isolates from

both IC and IP and the types of clinical specimens from which *Aspergilli* were isolated are shown in table 2.

Identification of *Aspergilli* done by direct microscopical examination of the clinical specimens by different staining methods in addition to the culture, slide culture technique and growth on Czapek's agar.

Ouchterlony (ID) test detected the *Aspergillus* antibody in serum of one patient out of the 12 with *Aspergillus* isolates in comparison to the control (Fig.1). Serum of 33 normal controls, and 33 cases of confirmed *Aspergillus* negative results were tested in the same manner for specific antibodies against *Aspergillus* antigens, and all showed negative results.

Miscellaneous opportunistic molds

Ten isolates of the filamentous fungi were detected. They were 4 (1.9%) *Penicillium*, 3 (1.5%) *Cladosporium*, 2 (1%) *Fusarium*, and 1 (0.5%) *Geotrichum* (Table 1). The identification depended on the direct microscopical appearance of oval cells and fragments of mycelial elements by different staining methods, in addition to the culture on different media for the colonial morphology and microscopy.

Table (1): The multicellular and unicellular opportunistic fungi isolated from patients.

Immunocompetent (no.=150)		Immunocompromised (no.= 150)		Total		Fungi
%	No.	%	No.	%	No.	
MULTICELLULAR						
2.0	4	8.8	18	10.7	22	Opportunistic
1.0	2	4.9	10	5.9	12	<i>Aspergillus spp.</i>
-	-	1.9	4	1.9	4	<i>Penicillium spp.</i>
1.0	2	0.5	1	1.5	3	<i>Cladosporium spp.</i>
-	-	1.0	2	1.0	2	<i>Fusarium spp.</i>
-	-	0.5	1	0.5	1	<i>Geotrichum spp.</i>
UNICELLULAR						
37.3	76	51.8	106	89.2	182	Opportunistic
35.3	72	47.6	97	82.8	169	<i>Candida spp.</i>
1.5	3	1.9	4	3.4	7	<i>S. cerevisiae</i>
0.5	1	1.9	4	2.5	5	<i>C. neoformans</i>
-	-	0.5	1	0.5	1	<i>R. rubra</i>
39.3	80	60.7	124	100.0	204	Total

Significant difference between total No. of opportunistic isolates from immunocompromised and immunocompetent patients according to Chi-square test ($p < 0.001$).

Table (2): The number and percentage of opportunistic fungi isolated from clinical specimens in both IC and IP patients.

No. of patients with both sputum and B. wash	Immunocompetent				Immunocompromised				Total		Isolates
	B. wash		Sputum		B. wash		Sputum		%	No.	
	%	No.	%	No.	%	No.	%	No.			
7 i.c*, 6 i.p*	7.8	16	30	62	11.2	23	39.7	81	82.8	169	<i>Candida spp.</i>
2 i.c	0.5	1	0.5	1	1.5	3	4.5	9	5.9	12	<i>Aspergillus spp.</i>
1 i.c	0.5	1	1.5	3			2.0	4	3.4	7	<i>S. cerevisiae</i>
			0.5	1	0.5	1	1.5	3	2.5	5	<i>C. neoformans</i>
1 i.c					1.0	2	1.5	3	1.9	4	<i>Penicillium spp.</i>
	1.0	2					0.5	1	1.5	3	<i>Cladosporium spp.</i>
					0.5	1	0.5	1	1.0	2	<i>Fusarium spp.</i>
							0.5	1	0.5	1	<i>Geotrichum spp.</i>
							0.5	1	0.5	1	<i>R. rubra</i>
17 (8.3%)	9.8	20	32.5	67	14.7	30	51.2	104	100.0	204	Total

* i.c = immunocompromised, i.p = immunocompetent.

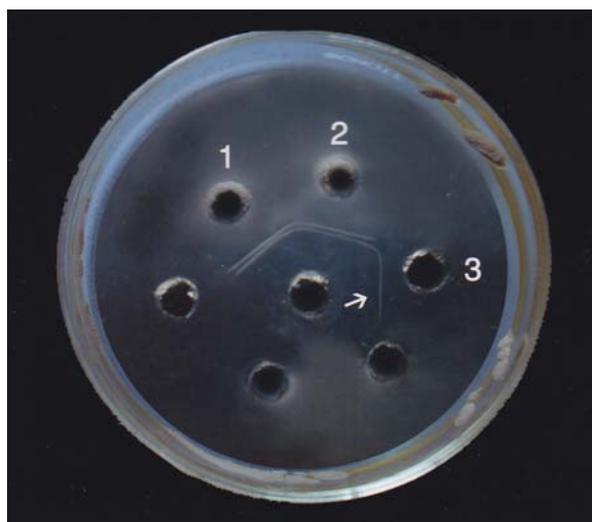


Figure (1): Ouchterlony test; central well contains *Aspergillus* antigen (neat concentration).

Wells 1 and 2 contain anti-*Aspergillus* antibody in neat concentration and 1/2 titer respectively as controls. Wells 3 to 6 contain sera from two patients with *Aspergillus* isolates (in similar concentration to wells 1 and 2):

Two lines of precipitate are seen for the controls at wells 1 and 2 (for *A. fumigatus* and *A. flavus*), while one line only of precipitate is seen for the first patient (positive for *A. flavus* isolate) in well 3 (arrowed).

Opportunistic unicellular fungi

The total number of yeasts isolated from cases of LRT infections was 182 (89.2%). These isolates belong to 4 genera namely Genus *Candida*, *Cryptococcus*, *Saccharomyces* and *Rhodotorula* (Table 1).

Genus *Candida*

The 169 (82.8%) isolates of this genus were categorized as *C. albicans* (129/169, 76.3%), *C. tropicalis* (14/169, 8.3%), *C. krusei* (5/169, 3%) and 21/169 (12.4%) were unidentified *Candida* species. The frequency of the isolated *Candida* species from IC were 47.6% in comparison to 35.3% in the IP ones (Table 1). The relation of *Candida* isolates to the clinical diagnosis showed that the higher percentages of *Candida* species were obtained from cases of pneumonia.

Different tests were used to identify the species of the 169 isolates including direct examination of the clinical specimens with different stains, and culture on different media. Additional identification tests namely germ tube, chlamyospore, and API-C system were also used.

Genus *Saccharomyces*

Seven isolates (3.4%) of the genus *Saccharomyces* were detected from both IC

(4) and IP (3) patients (Table 1). *Saccharomyces* grew well on modified Sabouraud's agar, and all the 7 isolates were identified as *S. cerevisiae* by API-C system.

Genus *Rhodotorula*

One isolate (0.5%) was obtained from the sputum of an IC patient (Table 2) with pneumonia. The isolate gave positive urease test and identified as *R. rubra* by API-C system.

Genus *Cryptococcus*

Five *Cryptococcus* isolates (2.5%) were detected from 4 IC and 1 IP patients (Table 1). For the identification, the appearance of the capsule in direct examination of the clinical specimen stained with nigrosine. Colonies grow well on BHI blood agar, in addition to their growth on modified Sabouraud's agar at 28°C, the isolates gave positive urease test and grew well at 37°C. All the detected Cryptococci were identified as *C. neoformans* by API-C system.

Discussion

It is not easy to determine the pathogenic role of fungal isolates from the respiratory tract, i.e., to differentiate between infections, colonization and contamination⁽¹⁶⁾. However, the prevalence and prognosis of pulmonary fungal infection has been difficult to evaluate since diagnosis was seldom confirmed⁽¹⁷⁾. A significant difference ($p < 0.001$) was recorded in this study between the opportunistic isolates from IC and IP patients. An important factor contributing to the increasing incidence of infection by fungi that have not been previously described to be pathogenic, is the rise in numbers of IC patients who are susceptible hosts for the most uncommon microbial agents⁽²⁾.

In order to validate the results of fungal isolation from patients, a similar identification process was carried out on the 50 individuals of the control group. It was found that all the fungi isolated were of opportunistic yeasts including *C. albicans* (14%), *S. cerevisiae* (2%), and unidentified *Candida* species (2%). This may throw light on the possibility of real LRT infections caused by the fungi isolated from patients.

Twelve patients showed *Aspergillus* isolates (5.9%). Those patients were predominantly males (11/12). This is in keeping with other studies that reported infection occurs in males more than females⁽¹⁸⁾. *Aspergillus* isolates were encountered more (10/12) in IC than IP group of patients. Since *Aspergillus* is an opportunistic fungus, the IC patients are more susceptible for its colonization. Whether infection (aspergillosis) could happen, it is a matter of balance between the immunity of the person versus the pathogenicity of the organism. Denning and Coworker⁽¹⁹⁾ mentioned that pulmonary aspergillosis depends upon the immune status of the patients. Airway colonization without evidence of tissue invasion can be found in chronic obstructive pulmonary disease patients, smokers, and even healthy individuals⁽²⁰⁾.

The first step in the evaluation of clinical specimens during this work is mounting in 20% KOH solution with or without calcofluor stain, and Gram's stain which are useful to assess the suitability of specimens for further processing and interpretation. Out of the 12 positive isolates of Aspergilli, 8 of them showed branching septate hyphae in the sputum, bronchial wash or both of them. Ellis⁽²¹⁾ reported that the presence of the hyaline, branching septate hyphae consistent with *Aspergillus* in any specimen from a patient with supporting clinical symptoms should be considered significant, but is not a specific identification of the causative agent. Immunodiffusion test showed positive line of precipitation for antibody in one case with *Aspergillus* isolate, while negative for antibody in the normal and case control groups. This test has proven to be of value in the diagnosis of aspergilloma and invasive aspergillosis. However, it should never be used alone, and must be correlated with other clinical and diagnostic data⁽²¹⁾.

The hyalohyphomycetes are rarely encountered in clinical specimens and rarely cause infection. Seven isolates of this group were identified during the study. All were obtained from IC patients with prolonged neutropenia, especially in leukemic patients, corticosteroid therapy, and cytotoxic

chemotherapy. It was reported that the infection was more frequent in patients with acute leukemia (56%) and most patients (83%) were neutropenic at diagnosis⁽²²⁾.

The one isolate of phaeohyphomycetes, namely *Cladosporium* was identified from 3 cases. The characteristic brown pigmented branching septate hyphae were detected in the clinical specimens in direct KOH and calcofluor mount, in addition to the culture. Clancy and Co-workers⁽²³⁾ reported that phaeohyphomycosis caused by brown-pigmented fungi where the tissue morphology of the causative organism are mycelia.

During recent years, a high incidence of yeast infection has been reported⁽²⁴⁾. In the present study, members of the 4 genera (*Candida*, *Cryptococcus*, *Saccharomyces*, and *Rhodotorula*) were identified from the clinical specimens. One hundred sixty nine (82.8%) isolates of *Candida* species were obtained. A significant higher percentage of *Candida* isolates (47.6%) were detected in IC in comparison to 35.5% in IP patients. Out of the 50 individuals included in this study as a control group, 16 (34%) of them showed *Candida* species in their sputa (14; 32% *C. albicans* and 2 (4%) unidentified *Candida* species. Nicod and Co-workers⁽⁹⁾ mentioned that a small number of *Candida* species are normally present in healthy persons, but increased when the normal microbial flora is altered by antibiotics or when there is a defect in immunocompetence. In this work, *C. albicans* (76.3%) is the main isolate in comparison to *C. tropicalis* (8.3%) and *C. krusei* (3%), in addition to 12.4% unidentified *Candida* species. Jaffer⁽¹⁸⁾ in Babylon province reported that *C. albicans* (69.2%) was the most common isolate in a study of pulmonary fungal infection, less frequency are *C. tropicalis* (19.2%), *C. kefyr* (7.6%) and *C. krusei* (3%).

One hundred twenty nine isolates of *C. albicans* reported in this study were identified by the production of germ tube and formation of chlamydospore except 4 isolates showed negative germ tube production and identified by API-C system as the other non albican candida species. Germ tube and

chlamydospore formation test were used for the identification of more than 90% of *C. albicans*⁽⁶⁾.

Cryptococcus identified in this study represent 2.3% of the isolates. Among the 5 patients with *Cryptococcus* isolates, 4 of them from IC. This illustrates that colonization or infection occurs predominantly in such patients. Furthermore, patients with pneumonia showed most of the *Cryptococcus* isolates (3/5). In a previous report, it was mentioned that *Cryptococcus* causes primarily pulmonary pneumonia then disseminates to other organs mainly the brain and meninges in IC patients⁽²⁵⁾. The identification of *Cryptococcus* depends on the presence of capsule, growth on BHI blood agar at 28°C and positive urease test, then identified to species level by API-C system. The demonstration of encapsulated yeast cells by nigrosine in sputum specimens should be considered significant for *Cryptococcus*⁽²⁴⁾.

Saccharomyces has been reported rarely as a cause of opportunistic infection. *Saccharomyces cerevisiae* which is identified by API-C system during the study, accounted for 3.4% of all the isolated fungi. Kiehn and colleagues⁽²⁶⁾ reported that *Saccharomyces cerevisiae* accounted for less than 1% of all yeast infections isolated at a cancer hospital and mostly isolated from the respiratory tract. *Saccharomyces cerevisiae* constitute part of the normal or transient flora of the throat⁽²⁷⁾ and from the control group of this study 2/50 (4%) individuals showed *Saccharomyces cerevisiae* in their sputum.

The *Rhodotorula* may colonize humans, but may infect individuals with predisposing risk factors. One isolate of this yeast was detected from sputum of IC patient, and identified as *R. rubra* by API-C system. Tuon and Costa mentioned that *Rhodotorula* was previously considered non-pathogenic, but during the last decades, it has emerged as an opportunistic etiologic agent particularly in IC patients⁽²⁸⁾.

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