

**ISOLATION AND CHARACTERISATION OF CANDIDA SPECIES FROM OROPHARYNGEAL SECRETIONS OF HIV POSITIVE INDIVIDUALS**

IZOLACJA I CHARAKTERYSTYKA CANDIDA SPECIES Z WYDZIELINY Z JAMY USTNO-GARDŁOWEJ U HIV POZYTYWNYCH OSÓB

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**Abstract**

**Material and methods:** Oropharyngeal secretions were collected from 100 HIV positive individuals with CD4 counts less than 500, over a period of 4 months from June-September 2010. Samples were processed by standard methods for isolation of candida. Speciation was done by the color of growth on HiCrome agar, germ tube test, pellicle formation on SDA broth, chlamyospore production on CMA, growth at 45°C and growth on Pal's agar. **Results:** Out of 100, 63 samples were positive for fungal growth. Among 63, 17 samples yielded mixed growth and 46 samples yielded single isolates. Total isolates from 63 samples were 80. *C. albicans* was the predominant species both in mixed cultures (17 out of 34 mixed isolates i.e 50%) & single isolates (18 out of 46 samples i.e 39%) and also in total isolates 35 out of 80 isolates, (43.7%) followed by *C. tropicalis* (17.6%, 20.9%, 18.7%), *C. parapsilosis* (20.5%, 6.9% and 12.5%), *C. dubliniensis* (11.8, 8.7%, 10%) in mixed, single and total isolates respectively. *C. krusei* and *C. glabrata* were obtained as single isolates. More positive cases were seen in individuals with CD4 count less than 200/cumm. Cultures (17 out of 34 mixed isolates i.e 50%) and single isolates (18 out of 46 samples i.e 39%) and also in total isolates (43.7%) followed by *C. tropicalis* (17.6%, 20.9%, 18.7%), *C. parapsilosis* (20.5%, 6.9%, 12.5%), *C. dubliniensis* (11.8, 8.7%, 10%) in mixed, single and total isolates respectively. *C. krusei* and *C. glabrata* were isolated as single isolates. More positive cases were seen in individuals with CD4 count less than 200/cumm.

**Streszczenie**

**Materiał i Metody:** Wydzielina z jamy ustnej i gardła została zebrana od 100 osób HIV pozytywnych z CD4 poniżej 500, w okresie 4 miesięcy od czerwca do września 2010 roku. Próbkę przetwarzano przez standardowe metody izolacji Candida. Specjacja była badana poprzez ocenę koloru wzrostu na agarze HiCrome, test kiełkowania, tworzenie błonki na bulionie SDA, tworzenie chlamydozpor na CMA, wzrost w 45°C oraz wzrost na agarze Pal. **Wyniki:** Spośród 100, 63 próbki były pozytywne dla wzrostu grzybów. Wśród 63 próbek 17 dały mieszany wzrost i w 46 próbkach uzyskano pojedynczą izolację. Całkowita liczba izolacji z 63 próbek wynosiła 80. *C. albicans* była dominującym gatunkiem zarówno w hodowlach mieszanych (17 spośród 34 mieszanych izolacji tj. 50%) i pojedyncze izolacje (18 z 46 próbek, czyli 39%), w sumie 35 izolacji z 80 (43,7%), następnie *C. tropicalis* (17,6%, 20,9% i 18,7%), *C. parapsilosis* (20,5%, 6,9% i 12,5%), *C. dubliniensis* (11,8, 8,7% i 10%) w mieszanych, pojedynczych i odpowiednio łącznej liczbie izolacji. *C. krusei* i *C. glabrata* uzyskano w pojedynczych izolacjach. Więcej pozytywnych przypadków obserwowano u osób z CD4 poniżej 200/cumm. Kultury (17 z 34 izolacji mieszanych tj. 50%) i pojedyncze izolacje (18 z 46 próbek tj. 39%), a także łącznie izolacje (43,7%), następnie *C. tropicalis* (17,6%, 20,9% i 18,7%), *C. parapsilosis* (20,5%, 6,9% i 12,5%), *C. dubliniensis* (11,8, 8,7% i 10%) odpowiednio w mieszanych, pojedynczych i łącznej liczbie izolacji. *C. krusei* i *C. glabrata* wyodrębniono jako pojedyncze izolaty. Więcej pozytywnych przypadków obserwowano u osób z CD4 poniżej 200/cumm.

**Key words:** colonization; *C. albicans*; non albicans species; opportunistic infections; HIV positive individuals**Słowa kluczowe:** kolonizacja; *C. albicans*; non albicans species; infekcja oportunistyczna; HIV pozytywne osoby**Introduction**

*Candida* species colonize the mucosal surfaces of all humans soon after birth and the risk of endogenous infection is ever-present [1]. Carriage rate of candida spp. tends to increase with age. Candidiasis of the oral mucosa is a disease, recognized since antiquity which has gained renewed significance more recently as an infection frequently seen in AIDS patients and in other

conditions.

Two medical events have received the interest in fungal diseases in general and candida infections in particular. The first was the introduction of antibacterial drugs in the second half of twentieth century. These drugs, especially those having broad spectrum of activity, may act as predisposing factors for mycotic infections by causing imbalance of the host's natural micro flora in

favor of fungi, upon which they have no inhibitory activity. The second event was the increase in the prevalence of immunosuppressed patients during the last few decades, as a result of chemotherapy or disease – AIDS which led to a parallel increase in the incidence of candida infection in general and the less pathogenic *non candida albicans spp.* in particular.

Candida spp. are the fifth most common cause of blood stream infections and fourth common cause of nosocomial infections [1,2].

*C. albicans* is generally considered the major pathogenic among the candida spp. Although an increase in the prevalence of *non-albicans spp.* has been noted during the past decade, because of the extensive use of anti mycotic drugs particularly azoles, for prolonged periods. Therapeutic courses has led to changes in the relative prevalence of various candida spp. with a decrease in the proportion of *C. albicans* as the etiological agent of candidiasis and an increase in the proportion of *non albicans spp.* such as *C. glabrata* and *C. krusei* [3]. *C. glabrata* is associated with severe complications than other species [4].

The newly recognized *C. dubliniensis* is an opportunistic pathogen that has been linked to oropharyngeal candidiasis in HIV infected patients. *C. dubliniensis* is closely related to *C. albicans* and is normally found in culture with *C. albicans*.

Oropharyngeal candidiasis (OPC) occurs primarily in individuals with HIV. In general most date to date data suggest that CD<sub>4</sub>+T Helper cells are critical for host defence against infections. Clinically OPC is most common when CD<sub>4</sub> + T cell count drops below 200 cells/cu mm. A threshold number of CD<sub>4</sub> + T cells was required to protect oral cavity against infection by the commensal organisms [5].

## Material Methods

Oropharyngeal secretions from 100 HIV positive individuals who attended to ART center (with a complaint of Sore throat) during a period of 4 months from June to September 2010 were isolated.

We got approval from Ethics Committee, Andhra Medical College, Visakhapatnam to conduct the study. The copy of the Ethics Committee Approval was enclosed.

Oropharyngeal secretions (swab from posterior pharyngeal wall) were collected with sterile swab, processed to isolate candida species .

Commonest age group was between 21-30 Yrs (41%) & male patients predominate in all age groups.

Samples were processed by standard methods. Direct smears were prepared and Gram Staining was done to look for Gram positive budding yeast cells and pseudo hyphae.

Samples were inoculated on SDA with antibiotics and incubated at 28<sup>0</sup>C in BOD incubator for one week. SDA bottles were observed daily for growth and bottles which did not yield any growth after one week were considered as negative.

SDA bottles with growth were processed. The growth that showed Gram positive budding yeast cells on Gram's staining was further processed by germ tube test, inoculation in SDA broth, inoculation on cornmeal agar, HiCrome agar, Pal's agar, for speciation of candida. To differentiate *C .albicans* from *C. dubliniensis* growth was inoculated on SDA and incubated at 45<sup>0</sup>C.

Speciation was done by the following characteristics (Tab. 1).

Species	Color on Chrome agar	Germ Tube Test	Chlamydorpores on CMA	Pellicle on SDA broth	Growth at 45 <sup>0</sup> C	Growth paints age
<i>C. albicans</i>	Light green	+	+	-	+	
<i>C. dubliniensis</i>	Dark green	++	++	NA	-	
<i>C. tropicalis</i>	Purphe halo in agar dark blue green colour	-	-	Small Pellicle	-	
<i>C. parapsilosis</i>	Pale colour	-	Pine forest appearance	NA	-	Rough Colour
<i>C. krusei</i>	Pale pink centre with white edge rough,spreading colony	-	-	Thick Pellicle	-	
<i>C. glabrata</i>	Dark pink	-	-		-	

**Table 1. Showing properties of candida species**

## Results (Fig. 1-10)

Out of 100 samples 63 samples showed gram positive budding yeast cells in direct Gram's staining and the same number yielded growth on SDA.

Among these 63 samples, 17 samples yielded mixed growth & *C.albicans* was the common species in all mixed growths. Single isolates were obtained from the

remaining 46 samples. *C. albicans* & *C. parapsilosis* were isolated in combination in 7 cases followed by *C. albicans* ,*C. tropicalis* & *C. albicans* ,*C. dubliniensis* (Tab. 2). Total cases 17.

As single isolate *C. albicans* was found in 18 samples and *non albicans spp.* in 28 samples. Among *non*

*albicans*, *C. tropicalis* *C. krusei* were the predominate isolates (Tab. 3).

Total samples & isolates – 46.

Total isolates from 63 samples were 80 .Of total 80 isolates, *C albicans* in 35 samples (43.7%) followed by *C. tropicalis* (18.7%) *C. parapsilosis* (12.5%) [Tab. 4].

Of total 100 individuals, CD4 counts were less than 100 in 10 and all were positive for OPC. 15 persons showed counts between 101-200, 13 were positive among them.

Name of Isolates	No of Samples	Total isolate	SPP
<i>C. albicans</i> & <i>dubliniensis</i>	4	17(50%) & 4(11.8%)	17,4*
<i>C. albicans</i> & <i>C. tropicalis</i>	6	6(17.6%)	6
<i>C. albicans</i> & <i>C. parapsilosis</i>	7	7 (20.5 %)	7

**Table 2.** Showing no of mixed isolates and their percentage. Total samples 17. Total isolates in mixed growth 34

Candida Spp	No. of isolates	%
<i>C. albicans</i>	18	39.1
<i>C. dubliniensis</i>	4	8.7
<i>C. tropicalis</i>	9	20.9
<i>C. krusei</i>	7	16.2
<i>C. glabrata</i>	5	11.6
<i>C. parapsilosis</i>	3	6.9

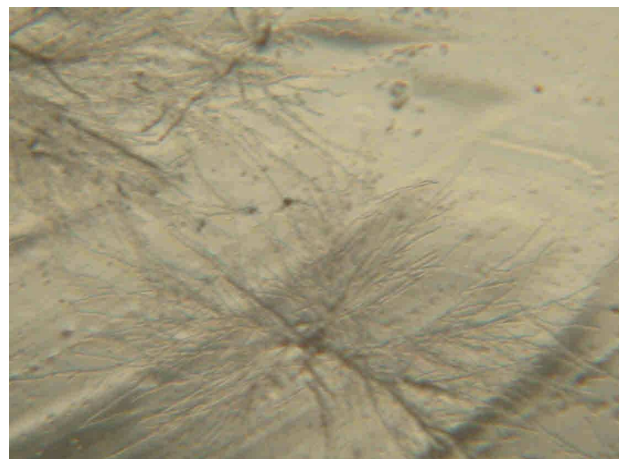
**Table 3.** Showing single isolates and their number. Total samples 46. Total isolates 46

Candida Spp	No. of Samples	%
<i>C. albicans</i>	35	43.7%
<i>C. dubliniensis</i>	8	10%
<i>C. tropicalis</i>	15	18.7%
<i>C. krusei</i>	7	8.7%
<i>C. glabrata</i>	5	6.25%
<i>C. parapsilosis</i>	10	12.5%

**Table 4.** Showing No. of Isolates in both single and mixed growth



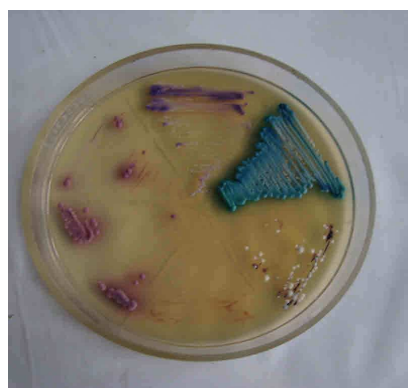
**Figure 1.** Growth of Candida In SDA



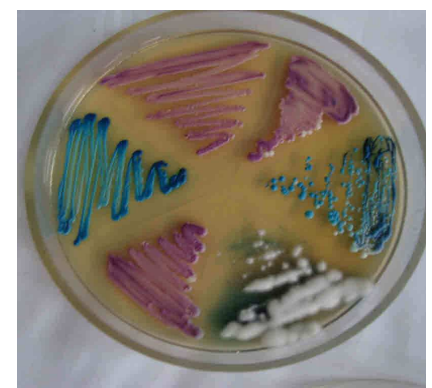
**Figure 2.** Pine forest appearance on CMA-C



**Figure 3.** Growth of Candida on HiCrome agar



**Figure 4.** Different colonies in HiCrome agar



**Figure 5.** *C. albicans*, *C. dubliniensis*, *C. glabrata*



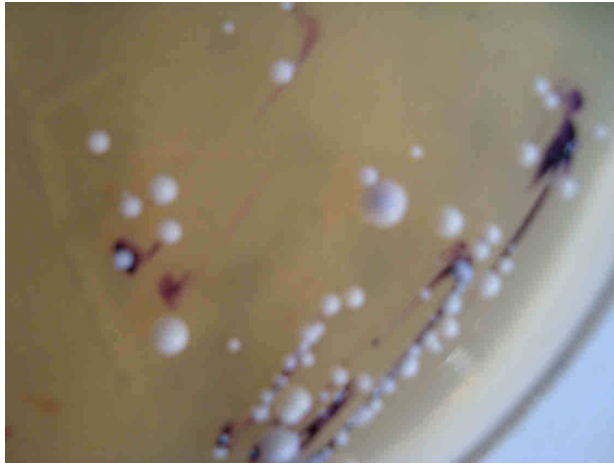


Figure 6. Pale colonies of *C. parapsilosis*

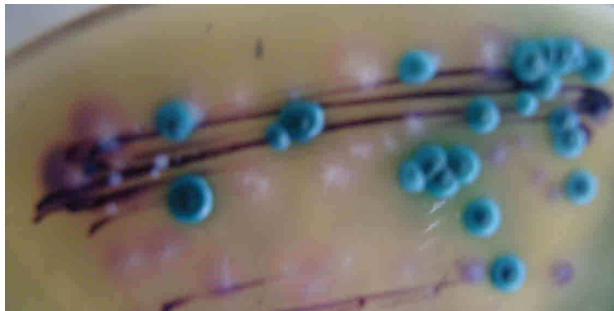


Figure 7. *C. albicans* and *C. tropicalis*



Figure 8. Light green colonies of *C. albicans*

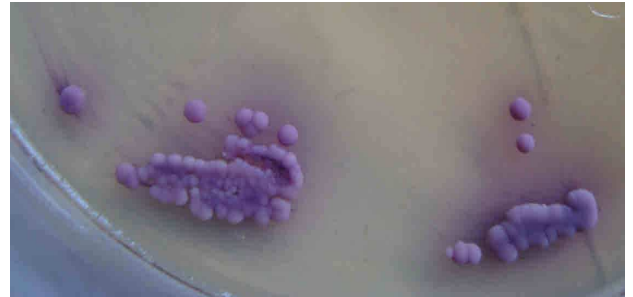


Figure 9. *C. tropicalis*

### Discussion

We know that colonization is the first step for disease (invasion preceded by colonization). Colonization leads to disease when conditions are favorable for eg. general factors like fall in CD<sub>4</sub> count, malnutrition, antibacterial therapy, DM etc. and local factors such as xerostomia and trauma from unhygienic or improperly fitted dentures etc. Saliva contains antifungal proteins including histatins and calprotectin that helps to protect from candida infection. These protective proteins absent in patients with Xerostomia [6,7,8]. As HIV patients are vulnerable to many bacterial diseases, treatment and prophylaxis with antibacterial agents is most frequent in them and make them predispose to fungal infections. So early recognition of fungal colonization and preventive measures like improvement in general health, oral hygiene, discriminate use of antibiotics may prevent fungal infections.

Today's concern about candidiasis is emergence of fluconazole resistant *C. albicans* in AIDS patients with recurrent attacks of oral thrush and less susceptibility of *C. krusei* and *C. glabrata* to fluconazole [6,8]. More over now *non-candida albicans* develop resistance to azoles for eg. *C. krusei* has known resistance to ketoconazole and *C. dubliniensis* has acquired resistance to fluconazole. OPC involves infections of hard and soft palate, buccal mucosa, gingiva and tongue. The infection can be atrophic with erythematous or pseudomembranous (thrush) with characteristic white lesions. Chewing and swallowing can be difficult under these conditions. Infections can be acute or recurrent and



Figure 10. Chlamydospores of *C. dubliniensis*

are common in immunocompromised patients especially those infected with HIV. Although OPC will occur under several immunosuppressed conditions, it appears to be much more common in HIV infected persons than any other conditions. Infact OPC is often one of the first clinical signs of underlying HIV infection and will occur in 50-95% of HIV positive persons some time during

their progression to full blown AIDS. Then it is possible that link between HIV & OPC is present that enhances susceptibility to OPC [5]. Clinically OPC is most common in HIV positive persons when CD<sub>4</sub> counts drops below 200cummm/ml as CMI plays an important role. In vitro immune analyses using peripheral blood mononuclear cells(PBMC) show that cells from most individuals respond to Candida antigens with Th<sub>1</sub> type cytokines. Then it is generally considered that susceptibility to OPC is enhanced under reduced CD4 T cells due to either lack of Th<sub>1</sub> type of response/shift to Th<sub>2</sub> type responses. Studies suggested that a threshold no. of CD4 cells are required to protect oral cavity against infection by this commensal organism. Below this threshold no. of cells, local immune mechanisms must function exclusively for protection. The prevalence of OPC may then depend on status of local immune mechanisms and non specific inhibitory factors like inhibitors in serum such as unsaturated transferrin and epithelial proliferation. As PMNC appear to play a role, neutropenic patients are susceptible to OPC [5,9]. Although *C. albicans* is most frequently isolated etiological agent, *C. glabrata*, *C. tropicalis*, *C. parapsilosis* and *C. guilliermondii* are also implicated in OPC [5]. Reviews have shown that candida esophagitis may occur frequently without thrush [1]. So by studying the prevalence of colonization of oropharynx among HIV the individuals, we can assess the risk of developing esophageal candidiasis. In a study from Cameroon, *C. albicans* is the only yeast isolated in oral swabs and it accounts for 73% [10]. Candidiasis was diagnosed in most cases of oropharyngeal lesions in 78 cases (about 50%) out of 160 cases in a study of opportunistic fungal infections in HIV/AIDS by Rakhmanova A [11]. In the present study out of 100 cases we isolated *C. albicans* in 17 cases as a mixed isolate and in 18 cases as a single isolate accounts for 43.7% as total isolates. *C. dubliniensis* is an opportunistic yeast that has been increasingly implicated in OPC in HIV infected patients. But because of its phenotypic similarities with *C. albicans*, *C. dubliniensis* is underreported. Moreover most *C. dubliniensis* isolates are susceptible to the fluconazole. The inducibility of azole resistance in vitro has been reported. Thus use of fluconazole prophylaxis in the treatment of HIV patients may have contributed to the increasing rates of isolation of *C. dubliniensis*. Martinez M used CHROM agar candida medium for initial isolation of *C. dubliniensis* and further identified by southern blot analysis with species specific probes Ca 3 (for *C. albicans*) Cd 25 (for *C. dubliniensis*). They isolated *C. albicans* from 42 cases of OPC and long term follow up for 3-6 months, *C. albicans* was replaced by *C. dubliniensis* in 8 cases whereas *C. albicans* failed to develop resistance to the fluconazole [12]. In the present study Hi Crome agar, growth at 45<sup>0</sup>C, CMA, were used to differentiate *C. dubliniensis* from *C. albicans*. *C. albicans* accounts for 43.7% isolates, *C. dubliniensis* accounts for 8.9% isolates in the present study. Usha Arora isolated 45 *C. albicans* and 15 non *C. albicans* species out of 60 candida isolates. Among non *C. albicans* spp. *C. tropicalis* was the commonest. The found low CD4 counts <200 Cummm in 46 patient [13]. The

Study of vp. Baradkar *et al.* Candida species were isolated from 50 patients out of 60 cases studied. *Candida albicans* was the commonest isolate (70 %) followed *Candida parapsilosis* (15%), *Candida glabrata* (7.5%) and *Candida tropicalis* (5%) respectively [14]. *Candida dubliniensis* was isolated from a single case only. In our study *C. albicans* accounts for 43.7% followed by *C. tropicalis*. 18.7%, *C. krusei* 8.7%, *C. glabrata* 6.25%, *C. dubliniensis* 10% and *C. parapsilosis* 6.9% (Tab. 4).

#### Conclusion:

As PCR is expensive and not available at all places, speciation of candida can be done by using HiCrome candida differential base, modified (HIMEDIA), growth on CMA for appearance of chlamyospore formation, growth at 45<sup>0</sup>c, growth on Pal's agar. In the present study though candida albicans is the major single isolate but non-albicans species were implicated in more number in OPC as a whole. *C. tropicalis* & *C. parapsilosis* were the next most common species after *C. albicans*. This work was done for the first time in and around Visakhapatnam, AP, India. Periodic study of fungal infection in HIV infected patients is the present day need, to know the changing pattern in incidence of candida species.

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