Identification of candida species isolated from oropharyngeal candidiasis of human immunodeficiency virus infected patients

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Abstract

Introduction: Oropharyngeal candidiasis (OC) is the most common opportunistic infection occurring in HIV infected individuals. Inappropriate use of antibiotics, antifungal agents and poor infection control practices has led to shift in spectrum towards non albicans candida species.

Materials and Methods: This is a prospective study for 6 months conducted at K.R Hospital, Mysore. HIV seropositive cases with oral candidiasis were included in the study. After informed consent, detailed demographic data was obtained from the study participants and two swabs were collected. One swab was used for gram staining and KOH mount. The second swab was immediately inoculated on two Sabouraud's Dextrose Agar (SDA) supplemented with antibiotics and incubated at 37ºC and 25ºC. Cream pasty yeast colonies on SDA were subjected to Germ tube test, morphology on Corn meal agar, sugar assimilation test and fermentation tests.

Results: Total of 67 patients were included in the study. Pseudomembranous type of OC was common. All the samples yielded the growth of Candida. *Candida albicans* was the commonest spp isolated but 68.1% of the isolates were non albicans candida. *C. tropicalis*, *C. guillermondii*, *C. dublinensis*, *C. parapsilosis*, *C. krusei* and *C. kefyr* were the non albicans candida spp isolated.

Conclusion: As some of the non albicans candida are intrinsically resistant to antifungal agents identification up to species level is important. Inappropriate treatment will lead to increased morbidity and mortality among these patients.

Keywords: Oropharyngeal candidiasis, HIV, Non albicans candida, *C. tropicalis*, AIDS, CD4 count.

Introduction

Acquired Immunodeficiency Syndrome (AIDS) the modern pandemic has been a public threat since its discovery in 1981. Human Immunodeficiency Virus (HIV) infection prevalence among adults (15 – 49 yrs) of India is estimated to be 0.26% i.e. 21.17 lakh people are living with HIV in India while incidence is around 86 thousand cases per year.[1] In 2015, 67.6 thousand deaths occurred due to AIDS in India. However, India has successfully achieved the 6th Millennium development Goal of halting the HIV epidemic.[1]

CD4+ cells guard the oral cavity from infections. The HIV targets the CD4+ cells and there is progressive decrease in the CD4+ cell count. Oral candidiasis (OC) is first reported when median CD4+ count is around 391 x 10^6cells/L.[2] The low absolute CD4+ T lymphocyte count has been cited as the greatest risk factor for the development of oral candidiasis and current guidelines suggest increased risk once CD4 T lymphocyte counts fall below 200 cells/ cumm.[3] Threshold number of CD4 cells are needed to guard the oral cavity from commensal invasion.[4]

The yeast like fungi Candida occurs as normal oral commensal in 20-50% of Indian population[5]. But in HIV infection the colonization rate increases dramatically to 81.3% on an average.[5] Increased intensity of colonization over time culminates in oral candidiasis. OC is a disease recognized since antiquity but gained significance more recently as an infection frequently seen in AIDS patients.[4] Use of antibiotics which cause imbalance in the microbial flora of the mouth can also favor fungal infections. The use of antifungal agents, mainly the azoles has increased prevalence of infections by Non albicans candida.[4]

OC is the first clinical sign in HIV infection and will occur in 50 – 95% of HIV positive persons some time during their progression to full blown AIDS.[4] Oral candidiasis being painful and recurrent, decreases the compliance for Antiretroviral Therapy (ART) and food intake.[6] Decreased compliance to ART further increases the viral load and the disease progresses among these patients.

C. albicans has been the most common candida species to cause OC. But among HIV infected individuals Non albicans candida are the common cause of OC. Non albicans candida spp exhibit intrinsic and acquired resistance to antifungals. The non albicans candida spp like *C. glabrata* and *C. krusei* are more resistant to Fluconazole and Itraconazole compared to *C. albicans*[7]. Some studies have reported resistance even to Amphotericin in 1.61% of candida isolates.[8] Coexistence of different species poses difficulty in treatment.[6] The changing susceptibility pattern of *C. albicans* and emergence of other species of Candida as pathogen have necessitated the identification of candida to species level.[9]

The present study was undertaken to isolate and identify candida in clinically suspected primary oral candidiasis among HIV seropositive cases.

Materials and Methods

This prospective study was conducted for 6 months after approval from the institutional ethics committee at ART Center, K. R hospital, Mysore.

Patients reactive for HIV 1 or 2 or both by Comb assay, Capillus and Tridot test (NACO recommended algorithm) with clinically suspected oral candidiasis of all age group and both sexes attending to ART Centre of K. R Hospital, Mysore were included in the study. Patients were explained in detail about the study and informed written consent was taken. Detailed history was obtained regarding name, age, gender, socio economic status, occupation, h/o tuberculosis, present complaints, previous similar episodes, antifungal treatment, HAART treatment, smoking and alcoholism that was recorded in a prescribed proforma. After the history the lesion in the oral cavity was examined and sample was collected under sterile conditions. The sample was collected and processed as per standard protocol.[10- 13]

Collection of sample: Two sterile cotton tipped wooden swabs moistened with saline were used to swab and scrape the lesion without touching any other structure in the oral cavity. Swab was collected after rinsing the oral cavity. Swabs were kept in sterile containers and transported immediately to the laboratory and processed.

Microscopy: One swab was used for direct microscopic examination by Gram stain and 10% KOH mount. Gram stain was examined for pus cells and Gram positive oval budding yeast like cells with/ without pseudohyphae. KOH mount was observed for budding yeast like cells with/ without pseudohyphae.

Culture: Sample from the second swab was inoculated on Sabouraud's Dextrose Agar containing Gentamicin & Chloramphenicol and 5% blood agar. Culture tubes were incubated at 37°C and room temperature and were observed daily for growth. All the samples yielded creamy white pasty growth by 24 – 48 hrs. Yeast like colony was subjected to Gram stain for confirmation. Other tests for identification and confirmation of candida used are germ tube test, Sugar assimilation and sugar fermentation test.

Germ tube test: Germ tube test was performed on all yeast like cells for presumptive identification of *C. albicans* and *C. dubliniensis.*

Inoculation on Cornmeal agar: All the yeast isolated were inoculated onto Cornmeal agar (CMA) by Dalmau plate method and incubated at 25°C for 2 – 5 days. Microscopically the arrangement of pseudohyphae, blastospores and chlamydospore were used to identify various species of candida. Most of the Candida had typical morphological arrangement and were identified.

Carbohydrate fermentation test: Sugar fermentation test was done to detect acid and gas production from 2% Glucose, Maltose, Sucrose, and Lactose with Andrade's indicator and durhams tube. Final results were obtained after incubation for 5 – 7 days at 25°C.

Table 1: Carbohydrate assimilation test for identification of various candida spp[11,14]

Candida species	Glucose	Maltose	Sucrose	Lactose
C. albicans	Acid with gas	Acid with gas	Acid	-
C. tropicalis	Acid with gas	Acid with gas	Acid with gas	-
C. guillermondii	Acid with gas	-	Acid with gas	-
C. parapsilosis	Acid with gas	-	-	-
C. krusei	Acid with gas	-	-	-
C. kefyr	Acid with gas	Acid with gas	Acid with gas	-/Acid with gas
C. glabrata	Acid with gas	-	-	-
C. dubliniensis	Acid with gas	Acid with gas	Acid	-
C. stellatoidea	Acid with gas	Acid with gas	-	-

Carbohydrate assimilation test: For sugar assimilation test, 4% discs were prepared of sucrose, glucose, maltose, raffinose, trehalose, xylose, cellobiose and lactose sugars.

Overnight growth of yeast like colonies were suspended in normal saline with McFarland 5 turbidity. Sterile swab was soaked and lawn cultured on yeast nitrogen base and incubated at 25°C for 7 days. Presence of growth around the disc indicates the assimilation of the carbohydrates. The pattern of sugar assimilation and fermentation were used for species confirmation as seen in table 1 and 2.

Table 2: Carbohydrate assimilation test for identification of various candida spp[11,14]

Species of Candida	Glucose	Maltose	Sucrose	Trehalose	Galactose	Lactose	Xylose	Cellobiose	Raffinose	Dulcitol	Melibiose	Urease
C. albicans	+	+	+	+	+	-	+	-	-	-	-	-
C. tropicalis	+	+	+	+	+	-	+	+	-	-	-	-
C. guillermondii	+	+	+	+	+	-	+	+	+	+	+	-
C. dubliniensis	+	+	+	+	+	-	-	-	-	-	-	-
C. kefyr	+	-	+	-	+	+/-	+	+	+	-	-	-

C. krusei	+	-	-	-	-	-	-	-	+/-	-	-	+
C. glabrata	+	-	-	+	-	-	-	-	-	-	-	-
C. lusitaniae	+	+	+	+	+	-	+	+	-	-	-	-
C. parapsilosis	+	+	+	+	+	-	+	-	-	-	-	-
C. stellatoidea	+	+	-	+	+	-	+	-	-	-	-	-

The CD 4 cell count were estimated by Flow cytometry using FACS Calibur (Becton Dickinson, San Jose, California).

Results

A total of 67 patients attending to ART centre tested positive for HIV by 3 tests as per NACO guidelines with signs and symptoms of oral candidiasis for the first time were included in the study. The demographic data of the participants are as seen in Table 3.

The mean CD4 count of the study participants was 129cells/µL. The most common presentation was pseudomembranous type of oral candidiasis with painful swallowing.

Table 3: Demographic characteristics of the study population

Characteristics		Number	Percentage
Sex	Male	37	55.2%
	Female	30	44.8%
Age	< 15 yrs	00	0%
	16 – 30 yrs	19	28.4%
	31 – 45 yrs	33	49.2%
	46 – 60 yrs	14	20.9%
	61 – 75 yrs	01	1.5%
Co infected with Tuberculosis	Yes	38	56.7%
	No	29	43.3%
CD 4 count	<200 cells/ cumm	56	83.6%
	>200 cells/ cumm	11	16.4%
Has habit of smoking	Yes	34	50.7%
	No	33	49.3%
Has habit of Alcohol consumption	Yes	31	46.3%
	No	36	53.7%

Gram stain and KOH mount showed budding yeast like cells with/ without pseudohyphae in 62.7% (42/67) and 76.1% (51/67) respectively. Overall in 80.6% samples (54/67) budding yeast like cells was observed in microscopic examination. In the present study 72 isolates were obtained from 67 study participants. Sample from 5 patients yielded multiple growth.

All *C. albicans* and *C. dublineninsis* produced germ tube in fresh human serum at 37°C by 2 hrs.

Most common species of candida isolated was *C. albicans* (31.9%) followed by *C. tropicalis* (23.6%), *C. guillermondii* (15.3%), *C. dublinensis* (11.2%). Others isolated were *C. parapsilosis*, *C. krusei* and *C. kefyr* were 6.9%. 6.9% and 4.2% respectively. The alarming observation was 68.1% isolated were Non albicans candida. The species were identified based on their appearance in CMA, sugar assimilation and sugar fermentation test. The appearance of *C. albicans* (Fig 1), *C. dublinensis* (Fig 2), *C. tropicalis* (Fig 3), *C. guillermondii* (Fig 4), C. parapsilosis (Fig 5), *C. krusei* (Fig 6) &*C. kefyr* (Fig 7) are seen below.

Table 4: Distribution of candida species in the study population

Species isolated from the samples	Number of isolates	Percentage
C. albicans	23	31.94
C. dubliniensis	8	11.11
C. tropicalis	17	23.62
C. guillermondii	11	15.28
C. parapsilosis	5	6.94
C. krusei	5	6.94
C. kefyr	3	4.17
Total	72	100

Correlation of oral candidiasis with CD 4 count showed a high significance with occurrence of OC among patients with CD 4 count < 200 cells/cumm ($p<0.001$).

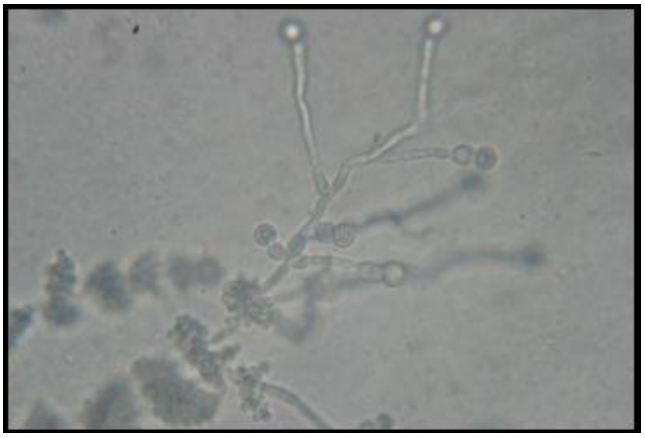

Fig. 1: Clusters of blastoconidia at septa, pseudohyphae with single terminal chlamydospore seen in *C. albicans* (40X)

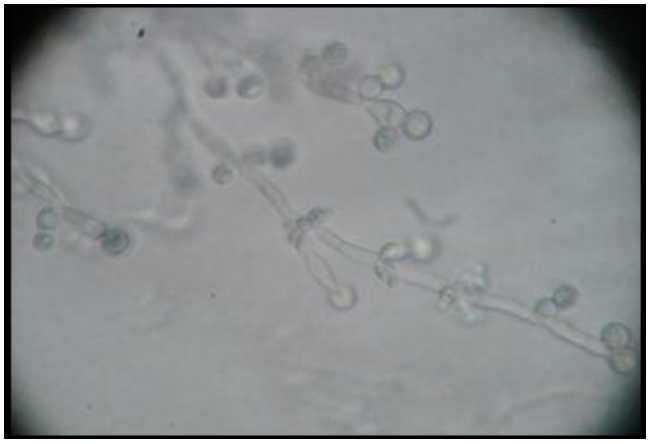

Fig. 2: Pseudohyphae with multiple terminal and intercalary chlamydospore seen in *C. dubliniensis* (40x)

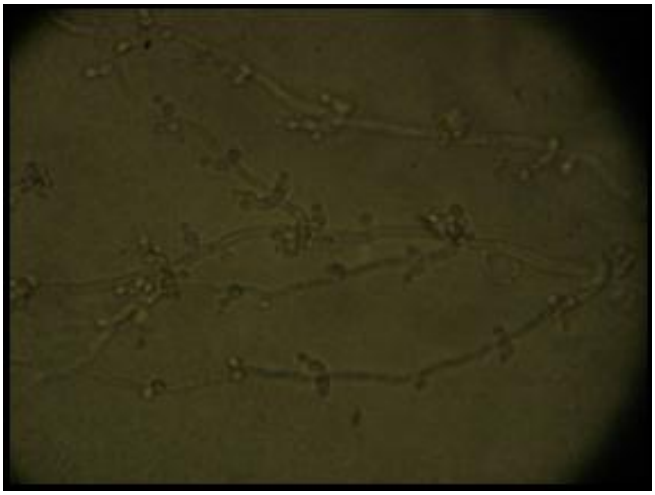

Fig. 3: Microscopic appearance of *C. tropicalis* shows Pseudohyphae with few blastoconidia at the septa (40X)

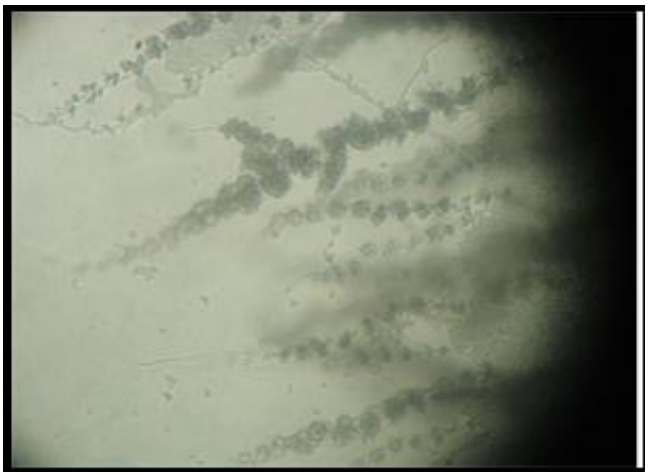

Fig. 4: Clusters of blastoconidia all along the pseudohphae seen in *C. guillermondii* (10X)

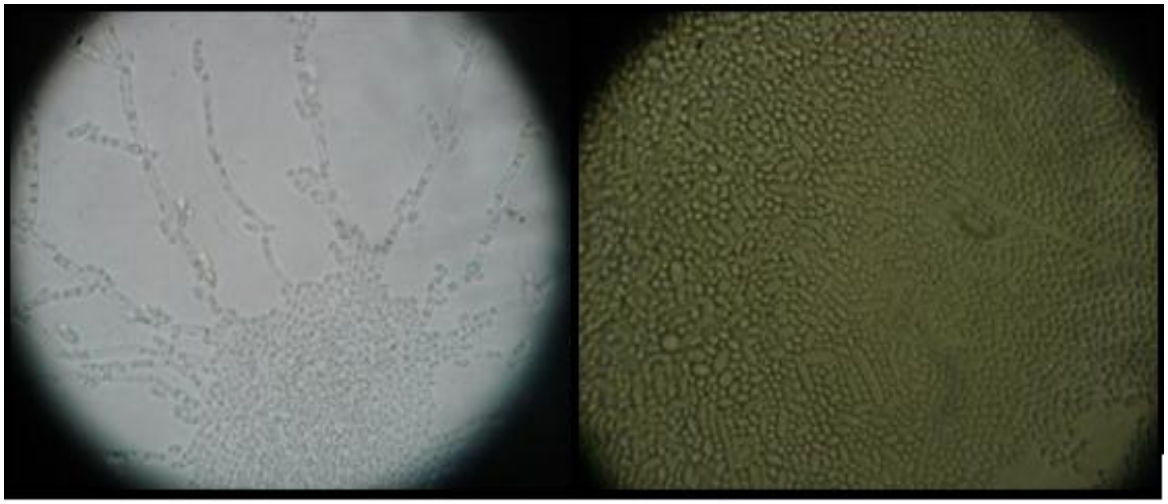

Fig. 5: Microscopic appearance of *C. parapsilosis* growth in CMA (40X) Spider like colonies along the streak line and Gaint cells with pseudohyphae seen

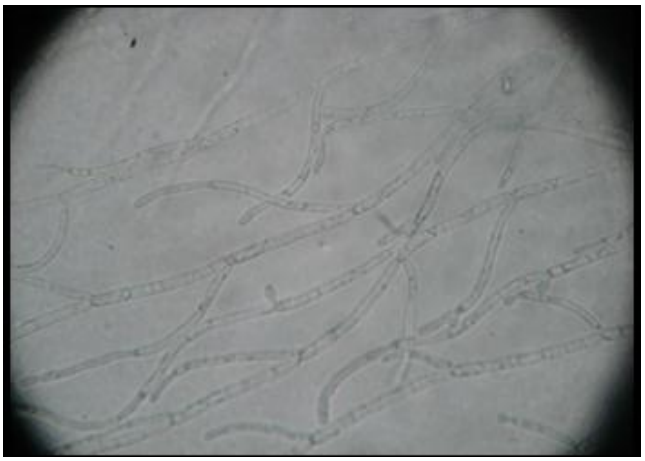

Fig. 6: Few elongated blastoconidia with plenty of pseudohyphae, cross matchstick appearance seen in *C. krusei* (40X)

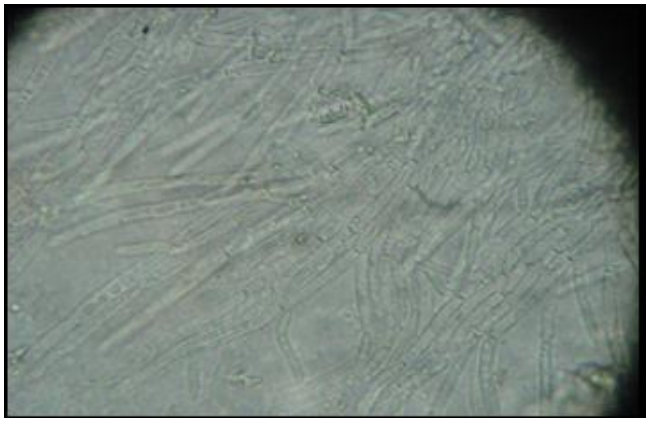

Fig 7: Elongated blastoconidia with pseudohyphae, log in stream appearance seen in *C. kefyr* (40X)

Discussion

Oropharyngeal candidiasis is the most common opportunistic fungal infection among HIV infected patients. Immunological status of the individual has a definitive impact on the severity of the disease as reflected by the association of occurrence of oral candidiasis with low CD4 counts[9].

The present study population included 55.2% males. Other studies also reported male preponderance in their studies.[4, 8,15,16] Half of the participants in the present study were between 31 – 45 yrs. Many other studies have also reported the disease common in the age 25 – 40 yrs [4,8,15-17] as HIV infection is most commonly seen in the sexually active age group.

Smoking history was found in 50.7% and alcohol consumption in 46.3% of the study population. Other study reported 31.9% of the study group gave history of alcohol consumption and 24.8% were smokers.[17]

In this study 56.7% of the patients were also detected to have TB which is much higher than a study reported from Assam[17] were 9.9% were coinfected with TB. Incidence of tuberculosis is very high in HIV infection. Hence all HIV infected are screened for presence of tuberculosis as they are highly prone.

In the present study 90% of the patients presented with painful swallowing and whitish pseudomembranous lesion. Similar findings are reported from other studies.[8,16,17,18]

Oral candidiasis is common among individuals with low CD4 count. The mean CD4 count of the study group was 129 cells/µL. Another study reported the mean CD4 count as 117 cells/cumm.[17] Other studies also reported OC among persons with CD4 cells less than 200cells/ cumm.[4] As the CD4 count decreases, the commensal flora turns opportunistic pathogen.

80.6% of sample was positive for budding yeast like cells on microscopic examination. A study at Chennai reported 57.3% of the samples showed the presence of yeast like cells with pseudohyphae in Gram stain.[8] Another study reported gram stain positive in 53% cases.[18] Hence KOH mount and Gram stain are very useful for rapid presumptive identification of oral candidiasis.

All the samples yielded growth (100%). Five of the samples yielded multiple species of candida. Similarly in another study from Hubli[18], all samples yielded growth of candida of which three samples also yielded mixed growth. Identification of mixed infection is important as treatment of these cases may be difficult.

Most common species isolated was *C.albicans* (31.9%). But the remaining 68.1% were non albicans candida. *C. tropicalis* (23.6%), *C. guillermondii* (15.3%), *C.dubliniensis* (11.2%) were the other isolates. Rare species like *C. parapsilosis*, *C. kefyr* and *C. krusei* were also isolated. The correlation between CD4 count and occurrence of non albicans candida species was also statistically significant ($p < 0.001$). Non albicans candida (87.5%) is also reported from another study.[16] *C. tropicalis* was the predominant species (23.14%), other isolated were *C. guillermondii*, *C. parapsilosis*, *C. kefyr*, *C. krusei*, *C. glabrata* and *C. albicans* isolated in 19%, 13.22%, 12.4%, 9.09%, 7.9% and 12.39% respectively.[16] Another study conducted at Vishakapatnum[4] reported 39% of the isolates were *C.albicans* and remaining being Non albicans candida of which *C. tropicalis* and *C. parapsilosis* was isolated among 20.9% & 20.5% respectively. Another study from Tamil Nadu[8] reported 56.45% of their isolates was *C. albicans* and 19.3% was *C. tropicalis*. A study conducted at Hubli[18] has reported *C.albicans* as 66.6% of the isolates and 33.3% of the isolates were non albicans candida. Of these 48.9% was *C. dublinensis*, 20% *C. krusei*, 11% *C. parapsilosis*, 8.9% *C. tropicalis* and 4.9% *C. guillermondii*. Previously C. albicans was the most common cause of OC but among HIV infected most of the studies have reported non albicans candida as the most common causative. Hence all isolates must be speciated as some have intrinsic resistance to the commonly used azoles.

C. dubliniensis can be differentiated from *C.albicans* by absence of growth at 45°C and xylose fermentation.[8] Our study reported 11.2% isolates as *C. dubliniensis* which was higher than reported in other studies[8]. Germ tube test can be

used for rapid identification of *C.albicans* & *C. dubliniensis.*

Some studies have reported *C. albicans* as the common cause of OC among HIV infected cases. A study conducted at Assam[17] reported *C.albicans*, *C.dubliniensis*, *C.parapsilosis* and *C.glabrata* in 77%, 14.7%, 3.2% and 3.2% respectively. Other studies from Amritsar[15] reported *C. albicans* as the common isolated species.

Immunosuppression may be responsible for emergence of non albicans Candida causing oral candidiasis. Immunocompromised patients are prone for recurrent bacterial infections, hence treatment and prophylaxis with antibiotics may predispose to fungal infections.[4] Majority of the oral Candidiasis in immunocompetent individuals is still caused by C. albicans.

Species identification is of therapeutic importance as some fungi are intrinsically resistant to the azoles.

Conclusion

The incidence of oral candidiasis due to non albicans candida is on the rise among HIV patients. Hence it is a must to identify candida up to species level, as some species are intrinsically resistant to fluconazole and resistance to fluconazole is on the rise among other species of Candida.

Limitation: Antifungal susceptibility testing was not done.

Conflict of Interest: None.

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