Species Identification And Antifungal Susceptibility Pattern Of Candida Isolates From Oropharyngeal Lesions Of HIV Infected Patients.

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Abstracts: Background &Objective: Oropharyngeal candidiasis (OPC) is a common feature associated with HIV infection. Over the past decade, reports have documented a shift away from C. albicans as a major cause of infection to non albicans Candida (NAC) species. Several NAC spp are inherently resistant to commonly used antifungal drugs. The objective of the present study was to investigate the distribution pattern of Candida spp. from HIV infected patients with OPC and evaluate its antifungal susceptibility pattern. Methods: A total of 192 HIV infected patients with oropharyngeal lesions (OPL) suggestive of candidiasis and 60 non HIV infected healthy individuals presenting without any OPL were included in the study.Swabs collected from the site of lesions were used for the demonstration and isolation of Candida. Speciation of Candida isolates was done and antifungal susceptibility testing was performed by the disc diffusion method. Results: Out of 192 HIV-infected patients with OPL, 179(93.2%) showed growth of Candida. Isolation of NAC species was higher than C. albicans. Azole resistance was more in NAC species as compared to C. albicans.Conclusions: NAC species has emerged as an important cause of OPC in HIV infected patients. The increased isolation rates of NAC species and a gradual shift in the antifungal susceptibility profile underlines the need of early and accurate diagnosis of infecting Candida spp along with antifungal susceptibility testing for selecting the most appropriate antifungal agent for therapy. [Deorukhkar S et al NJIRM 2012; 3(4) :86-90]

Key Words: Antifungal susceptibility testing, candidiasis, glucose methylene blue Mueller Hinton agar, NAC species, Oropharyngeal lesions

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Introduction In the past two decades, there has been a marked increase in the incidence of fungal infections. Fungi, once considered microbiological curiosities without pathogenic potential, have emerged as important opportunistic human pathogens [1]. This is thought to be the result of an increase in size of the population at risk such as cancer patients, HIV infected patients, transplant recipients and broad spectrum antibiotic therapy.

All HIV positive patients are susceptible to oral lesions at some point of their illness[2]. Oropharyngeal candidiasis (OPC) is a common feature associated with HIV infection. Four major clinical types of oral candidiasis have been reported: Pseudomembranous, erythematous (atrophic), hyperplastic and angular cheilitis. These types frequently appear in HIV infected subjects and are considered reliable markers of disease progression[3].

OPC contributes considerably to morbidity when it presents with pain or a burning sensation leading to subsequent poor nutrition or even invasive infections such as esophagitis or candidemia [4].

Although *C. albicans* remains the most frequent cause of candidiasis, over the past decade, reports have documented a shift away from *C. albicans* to non albicans Candida (NAC) species[5]. Several NAC spp are inherently less susceptible to commonly used antifungal drugs; hence their rapid identification is essential for appropriate therapeutic approach. Therefore there is a need for continuous surveillance to monitor trends in incidence, species distribution and antifungal drug susceptibility profiles.

Many reports have shown the presence of various species of Candida in different clinical presentations, but there is a paucity of studies concerning species identification and antifungal susceptibility pattern in HIV infected patients with OPC [6].

The present study was designed at a rural tertiary care hospital in western Maharashtra, with an aim to investigate the distribution pattern of Candida spp. from HIV infected patients with OPC and evaluate its antifungal susceptibility pattern.

Material and Methods: The present study is part of a PhD thesis and was approved by the Institutional Ethics Committee (Registration No.FN.32/2010).

A total of 192 HIV infected patients with oropharyngeal lesions suggestive of candidiasis who agreed for the collection of the samples were included in the study. Data on patient demographics, history of prior mycoses, antifungal treatment and medication was collected at enrolment. Patients without antifungal treatment in the preceding 6 months were eligible. The patients were informed about the method of sample collection.

HIV seropositivity was defined as those who had tested positive for HIV antibodies by any two of the following tests: ELISA\Rapid\Simple, as per the recommendations of the WHO [7]. Oral candidiasis was defined as presence of Candida spp in the oral cavity accompanied by symptoms and signs of inflammation\mucositis and \or presence of white plaques [4].

60 non HIV infected individuals presenting without any oral lesions were also included in the study as a control group to determine the prevalence of Candida colonization among healthy individuals. Oral yeast colonization was defined as a presence of yeast in the oral cavity in the absence of symptoms and signs suggestive of candidiasis [4].

Sample collection and processing: Two swabs were taken from the site of the lesion and symptoms of inflammation suggestive of oral candidiasis were documented. These included soreness, erythema, ulceration and the presence or absence of white plaques in the mouth. These swabs were processed in the Mycology section of our Department.

Of these two swabs, one was used for the preparation of smear for Gram staining, while the other was inoculated on Sabouraud's Dextrose Agar (SDA) slants containing 2 µg of gentamicin per ml and 0.5% cycloheximide. Additionally, samples were also inoculated on HICHROM Candida differential agar (CHROM agar) (Himedia laboratories Pvt Ltd Mumbai) to improvise species identification based on coloured colony morphology. SDA slants and CHROM agar plates were incubated at 37^0C for 48 hours.

Colonization in non HIV infected individuals was determined by asking the subjects to rinse their mouths with 20 ml of saline for 30 seconds. After vigorous shaking for additional 30 seconds, the rinse was serially diluted (1 in 2 with sterile saline) and 100 µl of each dilution was inoculated on SDA and CHROM agar and incubated at 37^0C for 48 hours.

Species identification: All the Candida isolates were subjected to germ tube test using normal human serum. Isolates were identified up to the species level on the basis of morphology on corn meal agar, growth on HICHROME Candida agar, carbohydrate fermentation, and assimilation pattern.

Antifungal agents: The antifungal agents used were – fluconazole (FC) (10 µg), itraconazole (IC) (10 µg), ketoconazole (KC) (10 µg). Antifungal discs were procured from Himedia Laboratories Pvt. Ltd Mumbai.

Antifungal susceptibility test: Antifungal susceptibility test was done by the disc diffusion method on glucose methylene blue Mueller Hinton agar (GM-MH). GM-MH was prepared by addition of 2% glucose and 0.5 µg of methylene blue to Mueller Hinton agar.

The inoculum was prepared by picking five distinct colonies of approximately 1mm from 24 hours old cultures grown on Sabouraud's dextrose agar (SDA). Colonies were suspended in 5ml of sterile 0.85% saline. This suspension was vortexed to adjust the turbidity yielding $1x10^6$-$5x10^6$ cells/ml and streaked on the entire surface of GM-MH agar. The antifungal discs were placed 24mm apart from each other. The plates were then incubated at 37^o C for 24 hours. If insufficient growth was observed after 24 hours, the plates were read after 48 hours. Zone diameters were interpreted as per the approved NCCLS (M44-A) guidelines [8]. The quality

control test was performed by using *C. parapsilosis* (ATCC 22019), *C. krusei* (ATCC 6258), and *C. albicans* (ATCC 90028).

Result: Out of 192 HIV infected patients with OPC, 89(46.35%) patients had pseudomembranous candidiasis (thrush). Pure growth of Candida was obtained on SDA and HICHROM agar from 179 (93.2%) patients. Figure 1 and 2 shows the details of isolates from healthy controls and HIV infected patients.

Figure: 1 Candida isolates from healthy controls.

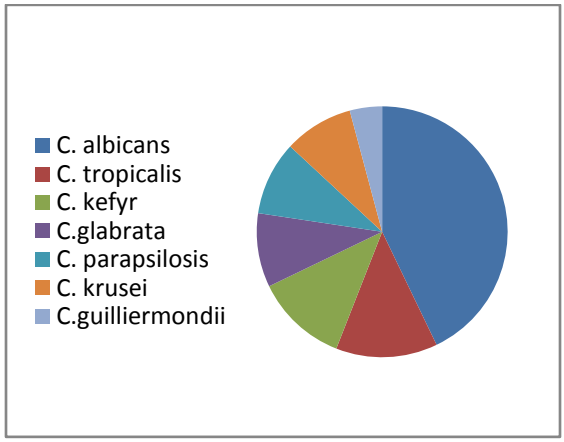
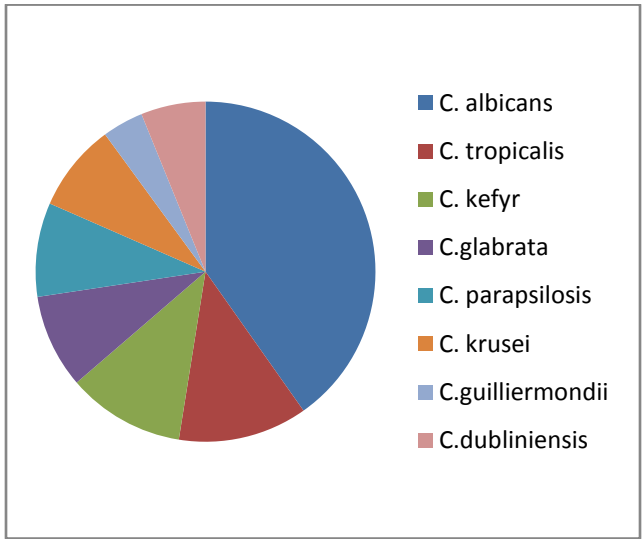


Figure: 2 Candida isolates from HIV infected patients.



The incidence of oral candidiasis due to NAC spp (59.7%) was more as compared to *C. albicans* (40.3%) among HIV infected patients. Among NAC species, *C. tropicalis* predominated. *C. dubliniensis* was isolated only from HIV infected patients.

Colonization was seen in 28(46.67%) healthy subjects (control group). 23 controls showed single Candida species whereas 5 showed growth of more than one species. In the control group *C.albicans* (57.1%) was the major isolate. Azole resistance was more in NAC species (Table 1). Maximum resistance to fluconazole was shown by most of NAC species.

Table: 1 Antifungal resistance patterns of Candida species isolated from HIV infected patients with OPC.

Candida species	Total	FC	IC	KC
C. albicans	72	04(5.55)	03(4.16)	06(8.33)
C. tropicalis	22	08(36.3)	07(31.8)	05(22.7)
C. kefyr	20	07(35)	05(25)	07(35)
C.glabrata	16	03(18.7)	02(12.5)	02(12.5)
C. parapsilosis	16	03(18.7)	03(18.7)	-
C. krusei	15	05(33.3)	03(20)	04(26.6)
C.dubliniensis	11	02(18.1)	02(18.1)	02(18.1)
C.guilliermondii	07	02(28.5)	02(28.5)	01(14.2)

FC: IC: KC:
Figures in parenthesis indicate percentage.

Discussion: The oral health status of an HIV infected patient at presentation is an extremely important parameter, as it may reveal important information regarding the immune status of the individual. Oral disorders occur in about 64% cases of HIV/AIDS in India. The most common oral disorder is OPC which occurs in 17-43% cases with HIV infection and in more than 90% of cases with AIDS [2].

In the present study, samples from 28 (46.67%) HIV non reactive subjects grew Candida spp. A total of 179 (93.22%) Candida spp were recovered from 192 HIV infected patients. Prevalence of NAC (59.8%) among HIV infected patients was higher than *C. albicans* (40.2%), whereas among HIV nonreactive subjects, 57.1% of the isolates were *C. albicans*. Previous studies show a higher rate of species variation in HIV positive patients

than HIV negative subjects. This variation may be due to several factors such as diet, oral hygiene, lack of access HAART, long term treatment with fluconazole etc [9].

Among NAC species *C. tropicalis* was the major isolate in HIV infected patients. Weinberg et al[10] and Ng et al[11]have also reported *C. tropicalis* predominance among NAC species. From the group of NAC species, *C. tropicalis* is becoming an emerging pathogen globally. Increasing number of immunocompromised patients along with increased use of an antifungal regimen and use of broad spectrum antibiotics are the major contributory factors for this [12].

C. dubliniensis was isolated only from HIV infected patients in the present study, which is similar to the observation of Chunchnur et al[13]. *C. dubliniensis* is emerging as a significant opportunistic pathogen in HIV infected patients, and is also drawing attention towards its ability to develop in vitro resistance to fluconazole.

With the increasing incidence of opportunistic fungal infection in HIV patients and the growing number of antifungal agents, laboratory aids to guide in the selection of antifungal therapy have gained greater importance.

The standardized broth micro-dilution method is expensive, laborious and cumbersome for routine use in clinical mycology services. Recently, a disc diffusion method has been recently approved by Clinical and Laboratory Standards Institute (CLSI). Disc diffusion procedure appears to be generally acceptable as a simple, in house standardized procedure for antifungal susceptibility of yeast [8].

In the present study we used GM-MH agar for antifungal susceptibility testing of Candida species isolated from HIV infected patients with oral candidiasis. Trailing phenomena around the zone margins were infrequent and minimal on the GM-MH agar. Zone edges with this method were frequently definite and clear, facilitating the measurement of zone sizes and minimizing subjectivity in zone size measurements. The occurrence of macro-colonies near the centre of the clear zone was also less with this method. The methylene blue in this medium stained the Candida colonies facilitating identification. Therefore GM-MH agar can be recommended as a simple, cost effective and sufficiently accurate medium for the routine testing of antifungal susceptibility of Candida spp.

Azole resistance was more common in NAC species as compared to *C. albicans*. Majority of the NAC species were resistant to fluconazole. The maximum resistance was seen in *C. tropicalis.* Other researchers have also noted an increase in the resistance to fluconazole in clinical isolates of *C. tropicalis* [14, 15].

Conclusion: To conclude, an increase in the resistance to antifungal agents by NAC species has made antifungal susceptibility testing essential for clinical microbiology services. For laboratories that do not perform broth micro-dilution susceptibility testing of yeast species on site, disc diffusion susceptibility testing using GM-MH agar can be adopted as a simple and reliable method to guide therapy.

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