

Original Research Article

Identification and In vitro Azole resistance of Candida species isolated from Oropharyngeal Candidiasis in Human Immunodeficiency Virus infected patients

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ABSTRACT

Keywords

Candida albicans, Oral thrush in HIV patients, Candida species

Oropharyngeal candidiasis continues to be a common opportunistic infection in patients infected with Human Immunodeficiency Virus (HIV). Though *Candida albicans* is the predominant isolate, rise in frequency of isolation of non albicans *Candida* (NAC) species is observed. Long term use of azoles for treatment as well as prophylaxis for Oropharyngeal candidiasis has lead to emergence of Azole resistant strains. The present study aimed at identification and characterization of *Candida* species and to derive their antifungal susceptibility pattern to Azole group of drugs from Oral candidiasis in HIV seropositive individuals. Oral swabs collected from 118 HIV seropositive patients were subjected for Gram stain and culture. Swabs yielding growth of *Candida* species are subjected to speciation by standard mycological methods like GTT, Dalmau-plate culture and sugar assimilation tests. Antifungal susceptibility testing done against Fluconazole, Voriconazole, Ketoconazole and Itraconazole by Disk Diffusion method according to CLSI guidelines (M44 A2). Out of 118 samples 121 species were isolated. NAC(87.6%) were the predominant isolates, *C. tropicalis*(23.14%) is the most common species isolated among NAC followed by *C. guilliermondii* (19%), *C. parapsilosis* (13.22%), *C. kefyr* (12.4%), *C. krusei*(9.09%), *C. glabrata*(5.79%), *C. fomata*(2.48%), *C. lusitaniae* (1.65%) and *C. pelliculosa* (0.83%) whereas *C. albicans* were 12.39%. *C. albicans* were 53.33%, 66.66% susceptible to Fluconazole and Voriconazole respectively whereas NAC showed 46.22%, 58.49% susceptibility to the same drugs. The present study underscores that, there is increased frequency of NAC in causing oral candidiasis in HIV seropositive individuals and decreased susceptibility to Fluconazole and Voriconazole among NAC than *C. albicans*.

Introduction

Over 40.3 million live with HIV, 4.9 million newly infected people and 3.1 million AIDS-related deaths occur globally (UNAIDS/WHO, 2005). HIV made a delayed entry into India, but its spread has

been very rapid and at present is in an advanced stage of the epidemic in some states of the country. Though HIV is the causative agent of AIDS, most morbidity and mortality in AIDS patients results from

opportunistic infections; approximately 80% of these patients are seen to die as a result of such an infection rather than from HIV. Limited studies from India have shown that tuberculosis is the most common opportunistic infection, followed by other bacterial, parasitic, viral and fungal infections. Various mycoses form the bulk of opportunistic infections in AIDS patients and are increasing in the form of an epidemic parallel to the AIDS epidemic (Wadhwa A et al.,2007).

Oropharyngeal candidiasis (OPC) is the most frequent opportunistic infection encountered in HIV infected individuals. It occurs in up to 90% at some point during the course of HIV disease. OPC certainly increases morbidity and mortality (Wadhwa A et al.,2007, Sánchez-vargas LO et al., 2005).

The advent of highly active antiretroviral therapy (HAART) has permitted suppression of viral replication and a partial recovery of CD4 T-lymphocyte count in HIV infected patients. Although the incidence and prevalence of opportunistic infections have been reduced worldwide due to use of HAART, OPC remains the most frequent HIV associated oral lesion in most developing countries. The prolonged course of HIV infection predisposes these patients to recurrent episodes of OPC that can increase in frequency and severity with progressive HIV disease (Sánchez-vargas LO., 2005).

Earlier, it was considered that *C. albicans* was the most common species causing oral candidiasis it is not so at present. Even though most respond well to a short course of azole therapy, up to 50% will experience a relapse within 1 month after the completion of therapy. Currently, the number of patients who experience multiple recurrences of mucosal Candidal infections

and eventually fail to respond to azole therapy is rising (Makarova NU., 2003).

The prolonged management of OPC might cause the development of drug-resistant OPC and there have been reports of emergence of resistance to antifungal agents in HIV/AIDS patients with OPC. OPC due to resistant *Candida* isolates fails to respond to antifungal treatment with appropriate doses for a standard duration of time. The other possibility is that, repeated exposure to antifungal agents mainly as Fluconazole prophylaxis may predispose to a shift to non albicans *Candida* species and associated refractory and recurrent infections (Hamza OJM., 2004).

Despite increasing reports of Fluconazole resistance in *C. albicans*, the investigations into the molecular mechanisms of resistance have been performed with relatively few isolates and all of them have exhibited stable azole resistance, and little is known about short-term or reversible resistance (Xlaogang He., 1994).

Antifungal drug resistance and the emergence of novel species and species previously not associated with human disease as potential pathogens have also greatly contributed to the drastic increase in oral candidiasis (Xlaogang He.,1994, Martinez M .,2002).

Hence the study was done to isolate *Candida* spp from oral candidiasis in HIV seropositive cases, speciate them by various tests and derive their anti-fungal susceptibility pattern.

Methods

The study was carried out during the year 2011-2012. Approval was obtained by the Institutional Research and Ethical Committee.

Total 118 HIV seropositive patients irrespective of duration of infection with oral candidiasis characterized by cream-white, curdy patches or erythematous lesions on dorsum of tongue/ buccal mucosa/ pharyngeal wall were included in the study (Walmsley S., 2001). Those patients who received antifungal treatment within one month duration was excluded (Riina Rautemaa., 2006). After taking written informed consent, specimens were collected by firmly swabbing the lesion with two sterile cotton swabs. One swab was used for direct smear and the other for culture on Emmon's modified Sabourauds Dextrose Agar (SDA) supplemented with antibiotics (gentamicin 5µg and chloramphenicol 50µg) plate, incubated at 37⁰C (Singh K., 1999). Cream coloured pasty yeast colonies on SDA were subjected to Germ Tube Test, morphology on Corn meal agar (Dalmau Plate Culture method) and Auxonographic sugar assimilation test incubated for 7 days for identification of yeasts up to species level (Walmsley S.,2001).

Germ Tube Test – A small portion of an isolated colony of the yeast to be tested was inoculated into the 0.5 ml human serum and incubated at 37⁰C for two hours. After two hours of incubation, a drop of the yeast serum suspension was placed on a glass slide, overlaid with a cover slip and examined microscopically for the presence of germ tube under low power microscope. Test said to be positive, if tube like extension from the parent cell half the width and three to four times the length and no constriction at the point of attachment to yeast cell is seen in >30% of total yeast cells within 2hrs of inoculation, the isolate was considered presumptively as *Candida albicans* or *Candida dubliniensis* (Barlow AJE., 1974).

Dalmau Plate Culture method - An isolated

colony from the primary culture media was ploughed into cornmeal agar plate containing 1% Tween 80, 45⁰ angles to the culture media. A sterile cover slip was placed over the surface of the agar, covering a portion of the inoculated streaks. The streak project beyond cover slip- provides partial anaerobic environment. Plate was incubated at 28⁰C for 48 hours.

Chlamydospore seen as large, highly refractile thick walled cell, single or multiple, terminal or intercalary seen in *C. albicans*. Based on typical morphological arrangement of pseudohyphae and blastoconidia as explained (Barlow AJE., 1974), species were identified.

Auxonographic sugar assimilation test - A yeast suspension was made from a 24-48 hrs culture grown in a sugar free media, in to 2 ml of Yeast Nitrogen base by adding heavy inoculums of 4MF. The suspension was added to 18 ml of molten agar cooled to 45⁰ c and mixed well and the entire volume was poured in to a 90 mm sterile petriplates. The plate was allowed to set at room temperature until the agar surface sets. With the help of sterile forceps, carbohydrate discs [Hi-media] dextrose, sucrose, lactose, maltose, Xylose, raffinose, trehalose was placed on the surface of the inoculated agar. The plates were incubated at 35⁰ C for 7 days. Colonies around the sugar disks suggests that the species has assimilated that particular sugar. Antifungal susceptibility test for the *Candida* isolates was done by Disk Diffusion method according to CLSI M44-A2 document. Mueller Hinton Agar supplemented with 2% glucose and 0.5µg/ml Methylene blue was used. Antibiotics tested were Fluconazole 25µg, Ketaconazole 10 µg, Itraconazole 10 µg and Voriconazole 10 µg (Pfaller M. A et al.,2004).

Result and Discussion

Of the total, 65 patients were within the age group of 26-50 years (mean age 44.25 years), predominant group were men (70%) with male female ratio 2.5:1. Most common clinical presentation was pseudomembranous type (92.3%) followed by erythematous lesions (5.93%).

Spectrum: Non albicans Candida (NAC) accounted for 87.6% of the isolates and *Candida albicans* accounted for 12.39%. *C. tropicalis* (23.14%) was the predominant isolate among NAC other species that were isolated included *C. guilliermondi* (19.01%), *C. parapsilosis* (13.22%), *C. krusei* (12.4%), *C. kefyr* (9.09%), *C. glabrata* (5.79%), *C. fomatata* (2.48%), *C. lusitaniae* (1.65%), *C. pelliculosa* (0.83%).

Three patients showed double infection (2.5%) with *C. glabrata* + *C. krusei*, *C. tropicalis* + *C. parapsilosis* and *C. fomatata* + *C. krusei*. There were no isolates of *C. dubliniensis*.

Invitro susceptibility results: The present study showed Fluconazole resistance of 58.6% followed by Voriconazole and Ketoconazole of 40.5 % respectively. Itraconazole showed maximum resistance of 65.2%. *Candida albicans* were more sensitive to antifungal drugs than Non albicans Candida (figure 1). Susceptibility to four drugs with respect to species is depicted in Table 1.

The spectrum of oral candidiasis has changed with emergence of Non albicans *Candida* spp. and acquired antifungal drug resistance assisted by high risk population. The predominance of Non albicans *Candida* over *C. albicans* was a notable feature with *C. tropicalis* as the commonest spp. This goes in agreement with other authors from

India and western countries (Wadhwa A et al., 2007, Hamza OJM et al.,2004, Xlaogang He et al.,1994). Use of Fluconazole for treatment of candidiasis, inappropriate antifungal usage for fungal infections, prophylaxis administered in high risk areas and poor infection control practices has led to shift in spectrum towards non albicans *Candida* species (Hamza OJM et al., 2004).

The present study showed overall higher resistance to Fluconazole as compared to previous studies (Wadhwa A et al.,2007,Makarova NU., 2003,Hamza OJM et al.,2004). Testing of Fluconazole depends on several factors, including culture medium composition, pH, inoculum concentration, method employed in addition to the level of resistance of the isolate. Disk diffusion method was used in our study which may be unable to differentiate fully resistant strains from those with dose-dependent susceptibilities, which may have been considered as resistant strains in our study. Azoles only have a fungistatic effect and therefore have allowed the emergence of multidrug resistance strains in patients (Dhamgaye S et al., 2012).

Fluconazole targets the fungal P450 cytochrome 14-alpha lanosterol demethylase encoded by the ERG11 gene and resistance is due to mutations which cause azole resistance in *Candida albicans*. Azoles target ERG11, either in cis by creating ERG11 alleles which encode a protein variant insensitive to azoles (Akins RA et al., 2005), or in trans by increasing the expression of ERG11 through gain of function mutations in the UPC2 gene, which encode a transcription factor regulating ERG11 (Barker KS at al.,2006).The second type acts by increasing the expression of membrane transporters which export the drugs and therefore decrease their intracellular concentration (Millon L et al.,1994).

Figure.1 Sensitivity pattern to antifungal drugs with respect to *C. albicans* and Non albicans *Candida*

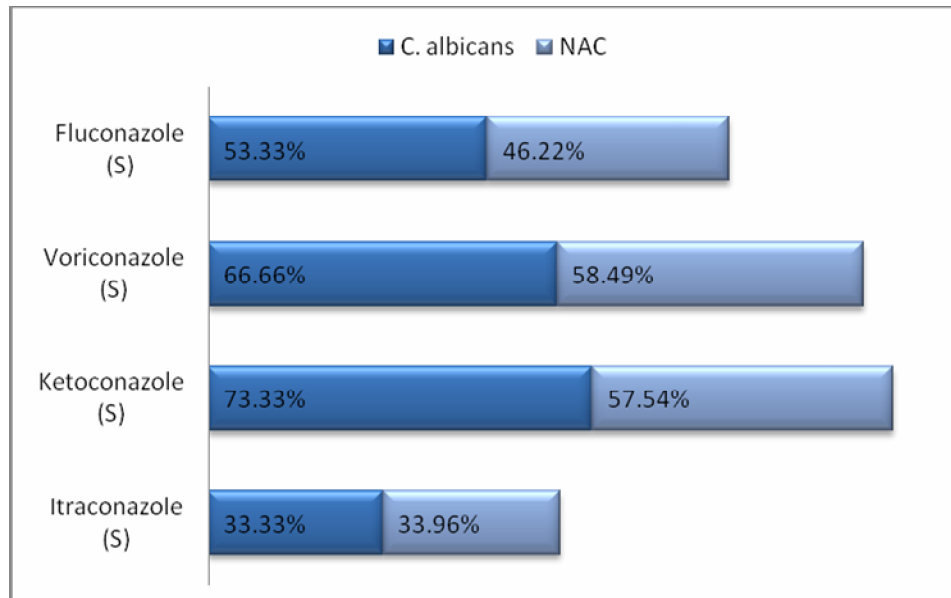


Table.1 Invitro susceptibility pattern with respect to species

Species	Fluconazole		Voriconazole		Ketoconazole		Itraconazole	
	R	S	R	S	R	S	R	S
<i>C. albicans</i>	46.6%	3.4%	3.4%	6.6%	6.6%	3.6%	6.6%	3.4%
<i>C. tropicalis</i>	60.7%	39.3%	39.3%	60.7%	39.3%	60.7%	57.2%	42.8%
<i>C. guilliermondii</i>	60.8%	39.2%	43.8%	56.2%	39.2%	60.8%	69.6%	30.4%
<i>C. parapsilosis</i>	75%	25%	50%	50%	37.5%	62.5%	62.5%	37.5%
<i>C. kefyr</i>	60%	40%	53.4%	46.6%	46.6%	53.4%	66.6%	33.4%
<i>C.krusei</i>	54.5%	45.5%	27.2%	72.3%	27.2%	72.3%	54.5%	45.5%
<i>C. glabrata</i>	57.1%	42.8%	28.5%	71.5%	71.5%	28.5%	100%	0
<i>C. fomata</i>	66.6%	33.4%	33.4%	66.6%	100%	0	100%	0
<i>C. lusitaniae</i>	50%	50%	50%	50%	50%	50%	50%	50%
<i>C. pelliculosa</i>	0	100%	0	100%	0	100%	100%	0

Two types of drug resistance, called MDR and CDR respectively, are distinguished, depending on the type of transporters which is involved (Dhamgaye S et al .,2012, Akins RA et al., 2005, Barker KS at al .,2006).

adapted for testing these drugs in the present study. Our study reveals higher resistance to Itraconazole (65.2%) and Ketoconazole (40.5%) and these results couldn't be correlated with clinical features.

No standards currently exist for invitro susceptibility testing of Itraconazole and Ketoconazole. CLSI proposed standards for Fluconazole susceptibility testing were

The present study analysis showed that oral infections with azole resistant *Candida* spp. occur more frequently among patients with certain identifiable risk factors: previous

azole therapy; advanced stage of HIV infection. Many of these factors appear inter-related. One hypothesis is that in advanced HIV infection there is a lack of response to azole therapy as a result of both profound T lymphocyte dysfunction and also dysfunction of other host defenses against *Candida*, such as neutrophil and monocyte fungicidal activity. As a consequence, the fungi are exposed to the drug for a prolonged period of time leading to induction of resistance or the emergence of pre-existing azole resistant strains (Millon L et al., 1994).

This study highlights the fact that there is potential risk of emergence and selection of azole resistant strains of *Candida* isolated from AIDS patients. This warrants careful selection of an antifungal drug for therapy of mild fungal infections after evaluation of in-vitro sensitivity of the isolated strains.

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