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Original Research Article

Species distribution, virulence factors and antifungal susceptibility profile of *Candida* isolated from Oropharyngeal lesions of HIV infected patients

Changdeo S. Aher*

Department of Microbiology, Dr. Vasantrao Pawar Medical College and Hospital and Research Center, Adgoan, Nashik, Maharashtra, India
Corresponding author

K e y w o r d s

Antifungal susceptibility testing; *Candida albicans;* extracellular hydrolytic enzymes; Non *albicans Candida* spp.

A B S T R A C T

Oropharyngeal candidiasis is a common feature associated with HIV infection. Although *Candida albicans* is considered to be most prevalent cause of mucosal and systemic infections, in recent years incidence of infections due to non *albicans Candida* (NAC) species has increased. The present study was conducted with an aim to determine species distribution, virulence factors and antifungal susceptibility profile of *Candida* spp. isolated from oropharyngeal lesions of HIV infected patients. A total of 148 *Candida* spp. isolated from oropharyngeal swabs collected from HIV patients with oropharyngeal lesions suggestive of oropharyngeal candidiasis were included in the study. The production of extracellular hydrolytic enzymes like phospholipase and proteinase were the virulence factors of *Candida* isolates studied. The antifungal susceptibility testing was performed by disc diffusion method. *C. glabrata* and *C. tropicalis* were the major isolates from NAC spp. Phospholipase production was seen in 49 (75.3%) isolates of *C. albicans*, 18 (60%) isolates of *C. glabrata* and 14(56%) *C. tropicalis* isolates. Proteinase production was noted in 35 (53.8%) *C. albicans* and 10 (40%) *C. tropicalis* isolates. Azole resistance was more common in NAC spp. as compared to *C. albicans*. This study underlines the importance of identification and antifungal susceptibility testing for selection of appropriate antifungal agent. The study of virulence factor like production of extracellular enzymes is necessary to understand the pathogenic role of infecting *Candida* spp.

Introduction

In recent years, incidence of mycotic infections has increased due to increase in number of patient with immunosuppressive viral infections and malignant tumors (Tanida *et al*., 2003). Among these infections Candidiasis is most common in this group of patients.

Oropharyngeal candidiasis (OPC) is a common feature associated with HIV infection (Deorukhkar *et al*., 2012 a). OPC occurs in approximately up to 90% of HIV infected cases during the course of infection (Hung *et al*., 2005). It is also considered as an important marker of HIV

and its progression (Mane *et al*., 2010). OPC increases morbidity and also negatively affect the quality of life of HIV infected patients.

The virulence of *Candida* spp. is attributed to certain factors like adherence, biofilm formation, and the production of tissue-damaging extracellular hydrolytic enzymes (Sardi et al., 2013). Extracellular hydrolytic enzymes like phospholipase and proteinase are important for colonization and invasion of host tissue (Sachin *et al*., 2012).

Although *Candida albicans* is considered to be most prevalent cause of mucosal and systemic infections, in recent years incidence of infections due to non *albicans Candida* (NAC) species like *C. glabrata*, *C. tropicalis* and *C. krusei* has increased (Deorukhkar and Saini, 2012 b). Infection due to NAC spp. is clinically indistinguishable from that caused by *C. albicans*, but are more resistant to routinely used antifungal drugs (Johnson *et al*., 1995). Therefore prompt identification of infecting species along with in vitro antifungal susceptibility testing is very important for prevention of emergence and spread of drug resistant *Candida* spp (Deorukhkar and Saini, 2013 a).

The present study was conducted with an aim to determine species distribution, virulence factors and antifungal susceptibility profile of *Candida* spp. isolated from oropharyngeal lesions of HIV infected patients.

Materials and Methods

The present study was carried out in the Department of Microbiology, Travancore Medical College, Kollam, Kerala. A total of 148 *Candida* spp. isolated from oropharyngeal swabs collected from HIV patients with oropharyngeal lesions suggestive of OPC were included in the study.

Candida spp. isolated was identified by standard mycological protocol including germ tube test, fermentation and assimilation of various sugars and colony color on Hichrom *Candida* agar (Deorukhkar and Saini, 2013 b).

The production of extracellular hydrolytic enzymes like phospholipase and proteinase were the virulence factors of *Candida* isolates studied.

Phospholipase activity of *Candida* spp. was determined by measuring the zone of precipitation on egg yolk agar (Samaranayake *et al*., 1984). The medium consisted of 13g of Sabouraud dextrose agar (SDA), 11.7 g Nacl, 0.11 g $CaCl_2$ and 10% sterile agar. The medium was prepared and inoculated by the method suggested by Deorukhkar and Saini (Deorukhkar and Saini, *et al*., 2014).

The phospholipase activity (Pz) was measured by the ratio of the diameter of the colony to the total diameter of the colony plus precipitation zone (Price *et al*., 1982). A P_z value of 1 denoted no phospholipase activity, $P_z<1$ indicated phospholipase activity. The lower the P_z value, the higher the phospholipase activity (Deorukhkar and Saini, 2013 b).

Proteinase activity of *Candida* spp. was determined by the method described by Staib (Staib, 1965). The bovine serum albumin (BSA) medium was used for screening proteinase activity of *Candida* isolates. The BSA medium consisted of dextrose 2%, KH_2PO_4 0.1%, $MgSO_4$ 0.05%, agar 2% and BSA solution 1%.

The medium was prepared and inoculated by the method suggested by Deorukhkar and Saini (Deorukhkar and Saini, *et al*., 2014).

Opaqueness of the agar corresponding to a zone of proteolysis surrounding the colony that could not stained with amidoblack indicated proteinase production by the isolate. The proteinase activity (Pr_z) was calculated in the terms of the ratio of the colony to the diameter of proteolytic unstained zone. A Pr_z value of 1 indicated no proteinase production, $Pr_z<1$ indicated proteinase activity. The lower the Pr_z value, the higher the proteinase activity (Deorukhkar and Saini, 2013 b).

The antifungal susceptibility testing for amphotericin B, fluconazole, itraconazole and ketoconazole was based on Clinical Laboratory Standards Institute (CLSI, formerly National Committee for Clinical Laboratory Standards (NCCLS)) disc diffusion method, 2004. Mueller-Hinton agar supplemented 0.2% glucose and 0.5µg/ml methyl blue dye was inoculated with a yeast suspension. The antifungal disc was then placed on the medium and the plate was incubated at 37^0 C. The zone size was measured after 24 hours and interpreted as per approved CLSI guidelines.

Results and Discussion

In the present study, out of 148 *Candida* isolates, 65 (43.9%) were identified as *C. albicans* and 83 (56.1%) were NAC spp. *C. glabrata* and *C. tropicalis* were the major isolates from NAC spp. 6 (7.2%) isolates from NAC spp. were identified as *C. dubliniensis* (Figure. 1).

The detail of phospholipase activity is shown in figure. 2, phospholipase production was seen in 49 (75.3%) isolates of *C. albicans*, 18 (60%) isolates of *C. glabrata* and 14(56%) *C. tropicalis* isolates.

Proteinase production was noted in 35 (53.8%) *C. albicans* and 10 (40%) *C. tropicalis* isolates. Proteinase activity was not seen in *C. parapsilosis* and *C. dubliniensis* (Figure. 3).

Table 1 shows antifungal susceptibility of *Candida* isolates. Amphotericin B resistance was seen in 14.9% of *Candida* isolates. Amphotericin B resistance was maximum in *C. glabrata* and *C. tropicalis* isolates. Amphotericin B resistance was not seen in *C. krusei* and *C. kefyr*. Fluconazole resistance was seen in 39.1% of *Candida* isolates. *C. tropicalis* followed by C. *dubliniensis* demonstrated maximum resistance to fluconazole. Itranazole resistance was seen in 39.1% of *Candida* isolates. *C. tropicalis* followed by *C. glabrata* showed maximum resistance to itraconazole. Ketoconazole resistance was seen in 37.2% of *Candida* isolates. *C. krusei* followed by *C. glabrata* showed maximum resistance to ketoconazole.

OPC is a common opportunistic fungal infection in immunocompromised patients (Li *et al*., 2007). However OPC is not associated with mortality but is a significant source of morbidity, and cause severe pain or discomfort upon mastication, which may limit nutrition intake (Redding *et al*., 2000).

C. albicans is considered as the major etiological agent of candidiasis including OPC. In recent years NAC spp. has emerged as notable pathogenic agents. They can cause oral lesion solely or in association with *C. albicans*. In the present study the predominance of NAC spp. as

Table.1 Antifungal susceptibility profile of *Candida* isolates.

Candida Spp.	Amphotericin B		Fluconazole		Itraconazole		Ketoconazole	
	S (%)	R(%)	S (%)	R(%)	S (%)	R(%)	S (%)	R(%)
C. albicans (n=65)	56 (86.2)	09 (13.8)	43 (66.2)	22 (33.8)	41 (63.1)	24 (36.9)	43 (66.2)	22 (33.8)
C. glabrata (n=30)	24 (80)	06 (20)	16 (53.3)	14 (46.4)	18 (60)	12 (40)	17 (56.6)	13 (43.4)
C. tropicalis (n=25)	20 (80)	05 (20)	12 (48)	13 (52)	11 (44)	14 (56)	14 (56)	11 (44)
C. krusei (n=09)	08 (88.9)	01 (11.1)	05 (55.5)	04 (44.5)	06 (66.6)	03 (33.4)	05 (55.5)	04 (44.5)
C. kefyr (n=7)	07 (100)	00	06 (85.7)	01 (14.3)	05 (71.4)	02 (28.6)	06 (85.7)	01 (14.3)
C. parapsilosis (n=6)	06 (100)	00	05 (83.3)	01 (16.4)	04 (66.6)	02 (33.4)	04 (66.6)	02 (33.4)
C. dubliniensis (n=6)	05 (83.3)	01 (16.4)	03 (50)	03 (50)	05 (83.3)	01 (16.4)	04 (66.6)	02 (33.4)
Total (n=148)	126 (85.1)	22 (14.9)	90 (60.9)	58 (39.1)	90 (60.9)	58 (39.1)	93 (62.8)	55 (37.2)

Figure.1 Species wise distribution of *Candida* isolates.

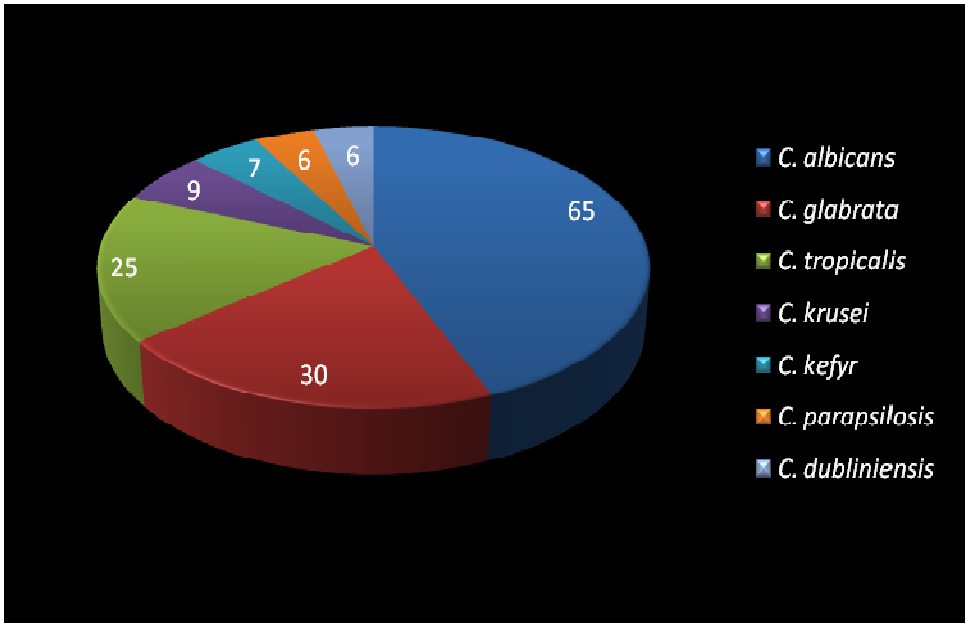


Figure.2 Phospholipase activity of Candida isolates.

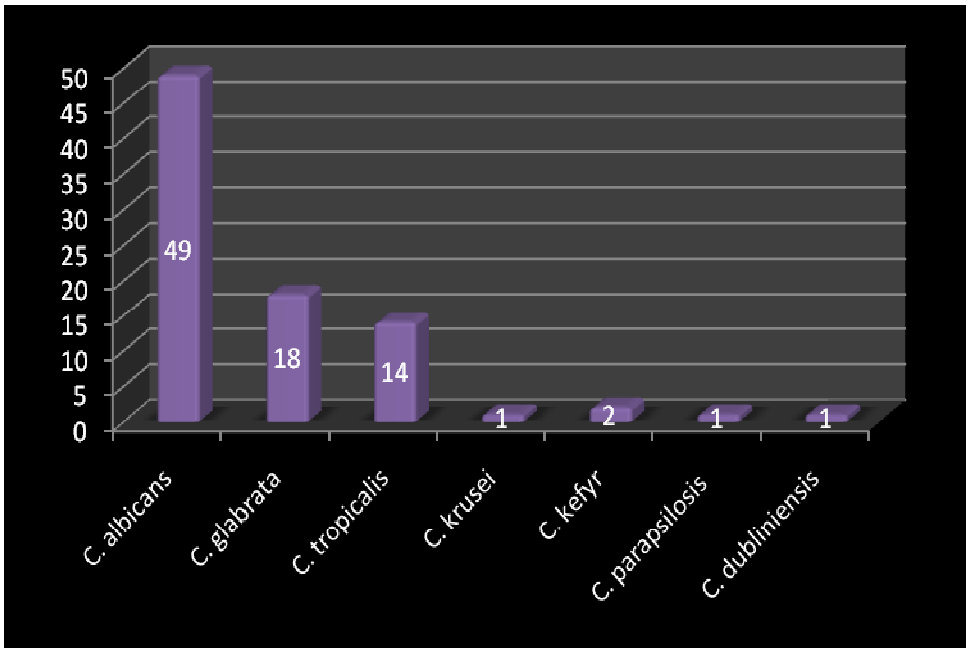
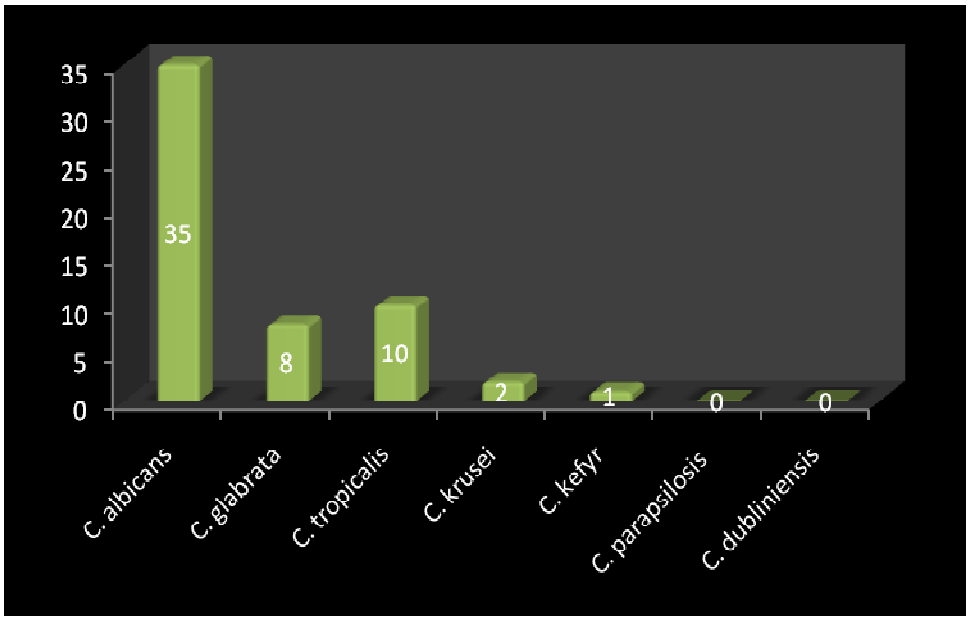


Figure.3 Proteinase activity of Candida isolates.



causative of OPC was noted. *C. glabrata* and *C. tropicalis* were the major isolates from NAC spp.*C. glabrata* currently ranks as the 2nd or 3rd most frequently isolated *Candida* spp. from various clinical types of candidiasis (Pfaller, 1996). The factors like the widespread use of immunosuppressive drugs and the emergence of HIV/AIDS have favored the increase of *C. glabrata* infections (Deorukhkar and Saini, 2014). OPC due to *C. glabrata* tend to be more severe and

more difficult to treat (Li *et al*., 2006). *C. glabrata* was the most frequent NAC spp. isolated from OPC in HIV-positive individuals in the study of Masia Conuto and co-workers (Masia Conuto *et al*., 2000). *C. tropicalis* was the second most common isolate from NAC spp. Our observation is in accordance to that of Deorukhkar et al (Deorukhkar *et al*., 2012 a). Increase in the population of immunocompromised hosts and use of broad spectrum antibiotics are precipitating factors for *C. tropicalis* infections (Kothavade *et al*., 2010).

Extracellular hydrolytic enzyme production is one of the virulence factors associated with the ability of *Candida* spp. to cause infections. Phospholipases are heterogeneous group of enzymes that hydrolyzes one or more ester linkages in glycerophospholipids. Its production helps the *Candida* to invade host tissue. In our study phospholipase activity was seen in 86 (58.1) isolates. Phospholipase production was maximum in *C. albicans* followed by *C. glabrata* and *C. tropicalis*. Four types of phospholipases (A, B, C and D) are reported to be secreted by *C. albicans* (Li *et al*., 2006). The phospholipase activity in *C. glabrata* is controversial, Samaranayake and co-workers in their study found that none of the *C. glabrata* isolates showed phospholipase production. In contrast to this finding, Deorukhkar and Saini reported phospholipase activity in 30.2% of *C. glabrata* isolates (Samaranayake *et al*., 1984; Deorukhkar and Saini, 2014).

Secreted aspartyl proteinases of *Candida* are capable of degrading epithelial and mucosal barrier proteins such as collagen, keratin and mucin, as well as antibodies, complement and cytokines (Borst and Fluit, 2003). In our study proteinase production was seen in 56 (37.8%) *Candida* isolates. Maximum proteinase activity was seen in *C. albicans* followed by *C. tropicalis* and *C. glabrata*. Proteinase production was not seen in *C. parapsilosis* and *C. dubliniensis*. The production of proteinase is recognized virulence attribute, facilitating tissue penetration (Wu *et al*., 1996).

The changing trend in species distribution and emergence of drug resistance in *Candida* has made antifungal susceptibility testing necessary. The CLSI standardized broth microdilution method is complex and laborious to use as a routine method. In the present study we used disc diffusion method for antifungal susceptibility testing of *Candida* isolates. Disc diffusion method is simple, cost effective and sufficiently accurate method (Deorukhkar and Saini, 2012 b).

In our study *Candida* isolates demonstrated more resistance to azole group of antifungal agents as compared to amphotericin B. Azole resistance in *Candida* spp. is of concern because these drugs are frequently used as therapeutic alternatives to amphotericin B (Deorukhkar and Saini, 2013 a). Amphotericin B has a rapid cidal action on most strain of *Candida* spp. but due to nephrotoxicity associated with it, amphotericin B is not the first choice of treatment (Giri and Kindo, 2012). Azole group of antifungal agents are preferred because they are easy for administration and are less toxic (Deorukhkar and Saini, 2013 a). Azole resistance was more common in NAC spp. as compared to *C. albicans*, which similar to the observation of Deorukhkar et al. (Deorukhkar et al., 2012 a)

From our study it can be concluded that the NAC spp. once considered as nonpathogenic commensal can cause OPC in HIV patient. Therefore identification and antifungal susceptibility testing is very important for selection of appropriate antifungal agent. The study of virulence factor like production of extracellular enzymes is necessary to understand the pathogenic role of infecting *Candida* spp.

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