Indian J Med Res 145, May 2017, pp 635-640
DOI: 10.4103/ijmr.IJMR_1773_14

Quick Response Code:

Estimation of biofilm, proteinase & phospholipase production of the *Candida* species isolated from the oropharyngeal samples in HIV-infected patients

Vicky Lahkar[1], Lahari Saikia[2], Saurav J. Patgiri[3], Reema Nath[2] & Partha Pratim Das[2]

[1]Department of Laboratory Medicine, Narayana Hrudayalaya Superspeciality Hospital, Guwahati, [2]Department of Microbiology & [3]Multidisciplinary Research Unit-ICMR, Assam Medical College & Hospital, Dibrugarh, India

Received December 2, 2014

***Background & objectives*: *Candida*, the most common opportunistic infection in acquired immunodeficiency syndrome (AIDS), attributes its pathogenicity to its virulence factors, mainly the biofilms, the proteinases and the phospholipases. There is a significant interplay of these factors during the HIV infection. This study was aimed to estimate the biofilm, proteinase and phospholipase production in *Candida* species isolated from the oropharyngeal samples in the HIV-infected patients.**

***Methods*: A total of 126 consecutive HIV-positive patients were screened for *Candida* growth using oropharyngeal swabs. Identification was done by Gram staining, germ tube test, chlamydospore identification, chromagar and biochemical tests on Vitek 2. Biofilm production was observed on Sabouraud's dextrose broth with glucose, phospholipase production in egg yolk agar medium and proteinase production in bovine serum albumin agar medium.**

***Results*: Of a total of 126 patients, 53 (42.06%) showed *Candida* growth: *Candida albicans* (n=46, 86.8%) was most common followed by the non-*albicans Candida* (NAC) (n=7, 13.93%). Of a total 33 (62.3%) biofilm positive isolates, significant production was observed in the NAC species ($P<0.05$). *C. albicans* reported the highest phospholipase (n=37/41, 90.24%) and proteinase (n=37/43, 86%) activities in a total of 41 (77%) phospholipase positive and 43 (81.1%) proteinase positive isolates.**

***Interpretation & conclusions*: Although *C. albicans* was the most common *Candida* species identified in HIV positive patients, the emergence of NAC was of special concern. Virulence factors such as biofilms, proteinases and phospholipases were noted in both these groups. Further research is required for better understanding of the pathogenic role of *Candida* species so as to aid in therapeutic interventions.**

Key words Biofilm - *Candida albicans* - HIV - non-*albicans Candida* - phospholipase - proteinase

The retrovirus - human immunodeficiency virus (HIV) 1 and 2 is responsible for causing the pandemic of acquired immunodeficiency syndrome (AIDS)[1]. Among the many opportunistic infections, oropharyngeal candidiasis is the most common and is an independent predictor of immunodeficiency in this condition[2,3]. The virulence of the *Candida* species is attributed to a wide variety of mechanisms

including production of biofilm, secretion of enzymes such as proteinases and phosphatases besides hyphae production and phenotypic switching[4].

The expression of these virulence factors may vary depending on the infecting species, geographical origin, type of infection, the site and stage of infection, and host reaction[5]. Therefore, the knowledge of these factors is important to understand the pathogenesis of candidiasis in HIV-positive patients and in addition, it will also help to explore new antifungal drug targets for improved therapeutic regimens. Various studies from India have explored these virulent attributes of *Candida* species[5-9]. However, studies are lacking from the northeastern part of India. Therefore, this study was aimed to estimate the biofilm, proteinase and phospholipase production of *Candida* species isolated from oropharyngeal samples in HIV-infected patients.

Material & Methods

A cross-sectional study on 126 consecutive patients was carried out in the department of Microbiology, Assam Medical College and Hospital, Dibrugarh, Assam, India, from April 2012 to March 2013. All patients who attended the Integrated Counselling and Testing Centre of Assam Medical College and Hospital during the study period and were found to be HIV seropositive, were included in the study and classified as per WHO clinical staging. HIV seropositive patients on highly active antiretroviral therapy (HAART) and HIV-seronegative patients were excluded. Informed written consent from the patients and ethical clearance from the Institutional Ethical Committee were taken.

Specimen collection and processing: Oropharyngeal swabs were collected from the patients under proper aseptic measures. The swabs were cultured in Sabouraud's dextrose agar (SDA) (HiMedia, Mumbai) with and without chloramphenicol (HiMedia). The organisms were isolated and identified by germ tube test, morphology on cornmeal agar (HiMedia), urea hydrolysis tests (HiMedia), chromagar media (HiMedia) and growth at 45°C, which were done as per standard microbiological protocols[10]. Vitek 2 YST identification card (bioMerieux, France) was also used for yeast identification as per manufacturer's instructions.

Determination of biofilm production: The method of Branchini *et al*[11] for *Candida* biofilm detection was used with modification. Briefly, a loopful of culture isolate from the SDA plate was inoculated into a tube containing 10 ml Sabouraud's liquid medium (HiMedia) supplemented with glucose (HiMedia). The tubes were incubated at 37°C for 24 h after which the broth was aspirated out, and the walls of the tubes were stained with safranin (HiMedia). Biofilm formation was scored as negative (0+), weak positive (1+), moderate positive (2+) or strong positive (3+). *Staphylococcus epidermidis* ATCC 35984 (American Type Culture Collection, Manassas, VA, USA) was used as the positive control and *S. epidermidis* ATCC 12228 as the negative control for biofilm detection and grading.

Determination of proteinase production: A modification of the method as proposed by Staib[12] was followed for detection of the proteinases. The yeast suspension (10 µl) was aliquoted into the wells punched onto the surface of the bovine serum albumin medium (HiMedia) and incubated at 37°C for two days. The plates were fixed with 20 per cent trichloroacetic acid and stained with 1.25 per cent amido black (HiMedia) followed by decolourisation with 15 per cent acetic acid. The plates were examined for opaqueness of the agar, corresponding to a zone of proteolysis around the wells. The diameter of unstained zones around the well was considered as a measure of proteinase production. The proteinase activity (Pz) was determined in terms of the ratio of the diameter of the well to the diameter of the proteolytic unstained zone. When Pz=1, no proteinase activity was detected in the strain. *Candida albicans* ATCC 10231 was used as a positive control.

Determination of phospholipase production: For phospholipase detection, the method proposed by Samaranayake *et al*[13] was used with modification. Aliquots of the yeast suspension (10 µl) were added to wells punched onto the surface of the egg yolk agar medium (HiMedia). The diameter of the precipitation zone around the well was measured after incubation at 37°C for two days. Phospholipase activity (Pz value) was determined as previously described[13]. When Pz=1, no phospholipase activity was detected in the strain. *C. albicans* ATCC 10231 was used as a positive control.

Statistical analysis: Data entry, database management and analysis were done using SPSS software, version 16.0 (SPSS Inc., Chicago, IL, USA). Statistical analysis was done by Chi-square test and Fisher's exact test wherever applicable.

Results

Of the 126 patients included in this study, the majority were in the 21-40 yr age group (n=86, 68.24%, $P<0.05$) with male preponderance (n=69, 54.7%). Most of them were educated up to 10th standard (n=115, 91.26%) and unemployed (n=81, 64.28%) and some of them had their own business (n=22, 17.46%). Rural population (n=90, 71.42%), mostly married (n=101, 80.15%, $P<0.05$) constituted most of the cases. History of alcohol intake (n=44, 34.92%) and smoking (n=41, 32.53%) were also seen. The HIV virus was mainly transmitted by the heterosexual route (n=81, 64.28%) with most of the patients presenting with World Health Organization clinical stage I disease (n=66, 52.38%) followed by stage III (n=34, 26.98%) and stage II (n=25, 19.84%). Seventeen patients (13.49%) suffered from HIV-tuberculosis co-infection with one patient presenting with *Cryptococcus* meningitis.

The mean CD4 count in the study group was 211.81±129.62 cells/µl with oropharyngeal lesions being observed in 28 patients. The presence of oropharyngeal lesions in patients with CD4 counts ≤200 cells/µl was found to be significant (n=21/33, $P<0.05$).

Of the 126 patients, 53 (42%) of the isolates were *Candida* isolates: *C. albicans* (n=46/53, 86.07%) was the most common followed by the non-*albicans Candida* (NAC) species, namely, *Candida parapsilosis* (5.66%), *Candida krusei* (3.77%), *Candida glabrata* (1.88%) and *Candida lipolytica* (1.88%) (Table).

Biofilm production was seen in 62 per cent (n=33/53) of the cases with a significant association ($P<0.05$) being observed in the NAC species. Its increased expression was also found to be significant ($P<0.05$) in patients with CD4 counts ≤200 cells/µl and with oropharyngeal lesions (Table).

Forty three isolates of 53 (81.1%) showed proteinase activity with *C. albicans* representing the major producer (n=37/43, 86%) followed by the NAC species. There was no association between the species of *Candida* isolated and the proteinase expression. However, significant association ($P<0.05$) was found with its expression in cases with CD4 counts ≤200 cells/µl and with oropharyngeal lesions (Table).

The production of phospholipase was seen in 41 cases of 53 (77%) and it was mainly produced by *C. albicans* (n=37/41, 90.24%). No significant association was found in the expression of this virulence factor to the species of the *Candida* isolated. However, with CD4 counts ≤200 cells/µl and the presence of oropharyngeal lesions, its increased expression was significant ($P<0.05$) (Table).

Discussion

In the present study, the biofilm, proteinase and phospholipase production of *Candida* species in the oropharyngeal swabs of the HIV-infected population was studied. Adherence of the fungus to host cells by biofilms initiates its colonization or disease[14,15]. NAC species was the major biofilm producer in the study, a finding in accordance with previous studies[6,7].

Extracellular phospholipase lyses host cells to facilitate adhesion and penetration[16]. Phospholipase enzyme breaks down the phospholipids of the cell membrane causing cell lysis, thereby direct host cell damage and lysis has been proposed as a major mechanism contributing to microbial virulence[16-19]. *C. albicans* was found to be the most frequent phospholipase producer followed by the NAC species namely *C. krusei, C. parapsilosis* and *C. lipolytica.* This finding was similar to that reported earlier[8,20].

Secreted aspartic proteinases damages the surface proteins (albumin, keratin) and degrades the locally protective IgA and C3 component[21,22]. This facilitates tissue invasion and resistance to the antimicrobial attack by the host[21]. The highest proteinase expression was seen in *C. albicans* in our study, followed by the NAC species as has been reported in previous studies[8,20].

The CD4 count denotes the immune status of the individual[1]. With decreasing CD4 counts (CD4 counts ≤200 cells/µl), the expression of biofilm, proteinase and the phospholipase increased. This has been shown by Jin *et al*[15] and De Bernardis *et al*[23]. The reason may be due to the fact that the preferential selection of strains with a higher overall level of these virulence factors increases with the advancing stages of HIV infection[9,24].

Oropharyngeal candidiasis is the most common manifestation of AIDS[2,3]. Its presence indicates the inability of the body's immune system to combat the microbe[2]. However, it indirectly leads to xerostomia facilitating increased cell adherence and thereby aiding biofilm formation[15,25]. Increased cell adherence also allows the phospholipases and the proteinases to act on the membrane phospholipids and target proteins, respectively.

Table. Association of virulence factors to *Candida* species isolated, CD4 cell count and oropharyngeal lesions

Variables	Biofilm						Proteinase						Phospholipase					
	Positive					*P*	Positive					*P*	Positive					*P*
	Strong	Moderate	Weak	Total	Percentage of all positive isolates		Strong	Moderate	Weak	Total	Percentage of all positive isolates		Strong	Moderate	Weak	Total	Percentage of all positive isolates	
Speciation																		
Candida albicans (n=46)	6	10	10	26	56.52	<0.05	12	19	6	37	80.43	NS	1	19	17	37	80.43	NS
Non-*C. albicans* (n=7)																		
C. parapsilosis (n=3)	1	1	1	3	100		0	1	2	3	100		0	0	1	1	33.33	
C. krusei (n=2)	0	1	1	2	100		1	1	0	2	100		0	1	1	2	100	
C. glabrata (n=1)	0	0	1	1	100		0	0	0	0	0		0	0	0	0	0	
C. lipolytica (n=1)	0	0	1	1	100		0	1	0	1	100		0	1	0	1	100	
CD4 count																		
≤200 cells/µl (n=29)	7	8	8	23	79.31	<0.05	10	14	3	27	93.1	<0.05	1	11	14	26	89.65	<0.05
>200 cells/µl (n=24)	0	4	6	10	41.66		3	8	4	15	62.5		2	7	5	14	58.33	
Candida isolates from oral lesions																		
Present (n=28)	6	8	9	23	82.1	<0.05	11	14	2	27	96.4	<0.05	1	11	13	25	89.2	<0.05
Absent (n=25)	1	4	5	10	40		2	6	5	13	52		0	8	5	13	52	

NS, not significant

Three major virulence factors of *Candida* species were measured in HIV-infected individuals. In this study, almost 30 per cent of *Candida* species associated with the oropharyngeal lesions were found not to produce any of these virulence factors. This finding was similar to the study by Kumar *et al*[26].

In conclusion, *C. albicans* was found to be the most common *Candida* species isolated from HIV infected patients. Biofilm production, proteinase and phospholipases were seen in both *C. albicans* and NAC. Further studies need to be done to understand the pathogenic role of *Candida* species in HIV positive patients and to device therapeutic modalities.

Acknowledgment

This study was funded by the Department of Biotechnology, Government of India (DBT Sanction No, BT/Mcd/l5/Vision-NER/2011, November 2, 2011). The authors acknowledge the Multidisciplinary Research Unit (ICMR), Assam Medical College, Dibrugarh, Assam, India for technical and infrastructure support. The authors also thank Shri Chakrapani Hazarika for statistical assistance and Shri Rajesh Sonar for technical assistance during the study.

***Conflicts of Interest*:** None.

References

1. Ananthanarayan R, Paniker CK. Human immunodeficiency virus: AIDS. In: Kapil A, editor. *Textbook of microbiology*, 9th ed. Hyderabad: Universities Press; 2013. p. 571-4.
2. Cook GC, Zumla A. HIV/AIDS with an emphasis on Africa. In: Gilks CF, editor. *Manson tropical diseases of medicine*, 21st ed. London: British Council, ELST with Saunders; 2003. p. 401-22.
3. Akpan A, Morgan R. Oral candidiasis. *Postgrad Med J* 2002; *78* : 455-9.
4. Calderone RA, Fonzi WA. Virulence factors of *Candida albicans*. *Trends Microbiol* 2001; *9* : 327-35.
5. Deorukhkar SC, Saini S, Mathew S. Virulence factors contributing to pathogenicity of *Candida tropicalis* and its antifungal susceptibility profile. *Int J Microbiol* 2014; *2014* : 456878.
6. Mohandas V, Ballal M. Distribution of *Candida* species in different clinical samples and their virulence: Biofilm formation, proteinase and phospholipase production: A study on hospitalized patients in Southern India. *J Glob Infect Dis* 2011; *3* : 4-8.
7. Kumar CP, Menon T. Biofilm production by clinical isolates of *Candida* species. *Med Mycol* 2006; *44* : 99-101.
8. Mohandas V, Ballal M. Proteinase and phospholipase activity as virulence factors in *Candida* species isolated from blood. *Rev Iberoam Micol* 2008; *25* : 208-10.
9. Mane A, Kulkarni A, Risbud A. Biofilm production in oral *Candida* isolates from HIV-positive individuals from Pune, India. *Mycoses* 2013; *56* : 182-6.
10. Forbes BA, Sahm DF, Weissfeld AS. Laboratory methods in basic mycology. In: Weissfeld AS, editor. *Bailey & Scott's diagnostic microbiology*, 12th ed. Missouri: Mosby Elsevier; 2007. p. 698-704.
11. Branchini ML, Pfaller MA, Rhine-Chalberg J, Frempong T, Isenberg HD. Genotypic variation and slime production among blood and catheter isolates of *Candida parapsilosis*. *J Clin Microbiol* 1994; *32* : 452-6.
12. Staib F. Serum-proteins as nitrogen source for yeast like fungi. *Sabouraudia* 1965; *4* : 187-93.
13. Samaranayake YH, Dassanayake RS, Jayatilake JA, Cheung BP, Yau JY, Yeung KW, *et al.* Phospholipase B enzyme expression is not associated with other virulence attributes in *Candida albicans* isolates from patients with human immunodeficiency virus infection. *J Med Microbiol* 2005; *54* (Pt 6) : 583-93.
14. Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: From the natural environment to infectious diseases. *Nat Rev Microbiol* 2004; *2* : 95-108.
15. Jin Y, Yip HK, Samaranayake YH, Yau JY, Samaranayake LP. Biofilm-forming ability of *Candida albicans* is unlikely to contribute to high levels of oral yeast carriage in cases of human immunodeficiency virus infection. *J Clin Microbiol* 2003; *41* : 2961-7.
16. Ghannoum M, Abu-Elteen KH. Pathogenicity determinants in *Candida*: A review. *J Mycol Med* 1990; *33* : 265-82.
17. Salyers A, Witt D. Virulence factors that damage the host. In: Salyers A, Witt D, editors. *Bacterial pathogenesis: A molecular approach*. Washington, DC: ASM Press; 1994. p. 47-62.
18. Leidich SD, Ibrahim AS, Fu Y, Koul A, Jessup C, Vitullo J, *et al.* Cloning and disruption of caPLB1, a phospholipase B gene involved in the pathogenicity of *Candida albicans*. *J Biol Chem* 1998; *273* : 26078-86.
19. Pugh D, Cawson RA. The cytochemical localization of phospholipase in *Candida albicans* infecting the chick chorio-allantoic membrane. *Sabouraudia* 1977; *15* : 29-35.
20. Oksuz S, Sahin I, Yildirim M, Gulcan A, Yavuz T, Kaya D, *et al.* Phospholipase and proteinase activities in different *Candida* species isolated from anatomically distinct sites of healthy adults. *Jpn J Infect Dis* 2007; *60* : 280-3.
21. Borst A, Fluit AC. High levels of hydrolytic enzymes secreted by *Candida albicans* isolates involved in respiratory infections. *J Med Microbiol* 2003; *52* (Pt 11) : 971-4.
22. Kaminishi H, Miyaguchi H, Tamaki T, Suenaga N, Hisamatsu M, Mihashi I, *et al*. Degradation of humoral host defense by *Candida albicans* proteinase. *Infect Immun* 1995; *63* : 984-8.
23. De Bernardis FV, Chiani P, Ciccozzi M, Pellegrini G, Ceddia T, D'Offizzi G, *et al.* Elevated aspartic proteinase secretion and experimental pathogenicity of *Candida albicans* isolates from oral cavities of subjects infected with human immunodeficiency virus. *Infect Immun* 1996; *64* : 466-71.
24. Ollert MW, Wende C, Görlich M, McMullan-Vogel CG, Borg-von Zepelin M, Vogel CW, *et al.* Increased expression of *Candida albicans* secretory proteinase, a putative virulence factor, in isolates from human immunodeficiency virus-positive patients. *J Clin Microbiol* 1995; *33* : 2543-9.

25. Chevalier M, Medioni E, Prêcheur I. Inhibition of *Candida albicans* yeast-hyphal transition and biofilm formation by *Solidago virgaurea* water extracts. *J Med Microbiol* 2012; *61* (Pt 7) : 1016-22.

26. Kumar CP, Kumar SS, Menon T. Phospholipase and proteinase activities of clinical isolates of *Candida* from immunocompromised patients. *Mycopathologia* 2006; *161* : 213-8.

Reprint requests: Dr Lahari Saikia, Department of Microbiology, Assam Medical College & Hospital, Dibrugarh 786 002, Assam, India
e-mail: mdrlamch@yahoo.com