SPECIES DISTRIBUTION AND IN VITRO ANTIFUNGAL SUSCEPTIBILITY PATTERN OF ORAL CANDIDA ISOLATES IN HIV PATIENTS AND CORRELATION WITH CD4 COUNT

Kalpana Devi V[1], Geetha Lakshmi S[2]

HOW TO CITE THIS ARTICLE:
Kalpana Devi V, Geetha Lakshmi S. "Species distribution and in-vitro antifungal susceptibility pattern of oral candida isolates in HIV patients and correlation with CD4 count". Journal of Evolution of Medical and Dental Sciences 2013; Vol2, Issue 47, November 25; Page: 9231-9238.

BACKGROUND: Oral Candidiasis, often the first sign of HIV infection, is the most prevalent fungal opportunistic infection in HIV infected individuals. The intrinsic resistance to antifungal therapy observed in some Candida species, along with the development of resistance during treatment in others, is becoming a major problem in the management of these diseases. Considering the above facts, the study was conducted to speciate the Candida isolates from HIV patients with Oral Candidiasis and to determine the antifungal susceptibility pattern and to correlate it with the CD4 count of the patients. **MATERIALS AND METHODS:** A total of 150 HIV patients with oral candidiasis were included in the study. Speciation of the Candida isolates were done using standard mycological techniques. Antifungal susceptibility of the Candida isolates for fluconazole and itraconazole was performed by microbroth dilution method as per CLSI guidelines. CD4 count of the patients were estimated using BD FACS counter and correlated with oral candidiasis. **RESULTS:** Candida albicans (77.6%) was the predominant species and the remaining were non albicans in the frequency of C. tropicalis (11.2%), C. krusei (5.3%), C. parapsilosis (3.9%), C. glabrata (1.3%), C. guilliermondii (0.7%). Azole resistance was more in non albicans candida species as compared to Candida albicans. Among the 150 HIV patients with Candidiasis, 106 (70.7%) patients had CD4 count < 200 Cells/µl. **CONCLUSION:** To conclude, though Candida albicans was the common species, the emergence of non-albicans Candida species and the increasing rate of azole resistance, emphasizes the need for speciation and determination of susceptibility pattern to provide appropriate treatment for HIV patients with oral candidiasis.

INTRODUCTION: Infection with Human immunodeficiency virus (HIV) and its end stage Acquired immunodeficiency syndrome (AIDS) is the major public challenge of modern times, with over 25 million persons already dead and over 30 million living with HIV / AIDS, the majority of whom are without access to therapy [1]. Opportunistic infection continue to cause morbidity and mortality in patients with human immunodeficiency virus infection throughout the world[2].

Oropharyngeal candidiasis (OPC) is the most common opportunistic infection observed in AIDS patients, occurring in an estimated 80 to 95% of these patients when the CD4 T lymphocyte counts are below 200 cells /mm^3 .Increased retroviral replication and an associated decline in immune defenses render these patients more susceptible to oropharyngeal candidiasis [3, 4, 5]. Although the introduction of antiretroviral therapy has had a major impact on the infectious complications of AIDS, candidiasis still remains a common opportunistic infection in HIV infected patients[6]. OPC is considered as one of the earliest indicator of HIV infection and is relatively reliable indicator marker of disease progression. Regardless of the CD4 count, OPC is predictive for the development of AIDS related illnesses if left untreated[7]. Candida albicans is the most common

species of yeast isolated from patients with OPC. The incidence of opportunistic infection due to C.albicans and other species has been increasing [8].

Antifungal drug resistance is fast becoming a major problem. Azole resistance is frequently described in patients with AIDS and mucosal candidiasis, oral candidiasis, or deep-seated candidiasis. Oropharyngeal colonization with Candida species was found in as many as 84% of patients infected with HIV, and symptomatic oral disease was found in up to 46% of these patients [9]. The high incidence of mucosal and deep seated forms of candidiasis has resulted in the use of systemic antifungal agents, especially fluconazole and itraconazole [10]. Many of these patients require long-term treatment to suppress oropharyngeal candidiasis. The widespread use of these antifungal agents have been followed by an increase in antifungal resistance and by a noticeable shift toward non albicans species with relative resistance to fluconazole and itraconazole [11] and there have been reports of emergence of resistance to antifungal agents in HIV/AIDS patients with OPC [12]. The increased reports of antifungal resistance and expanding drug therapy options prompted the need for clinically relevant antifungal susceptibility testing. Further the emergence of other Candida species such as Candida krusei and Candida glabrata with innately reduced susceptibilities to fluconazole [13] also results in treatment failure, emphasizing the need for speciation of the oral yeast isolates.

Considering the above facts, the present study was conducted to determine the species distribution and antifungal susceptibility profiles of the Candida isolates from HIV patients with Oropharyngeal Candidiasis.

MATERIAL AND METHODS: The present study was carried out at the Madras Medical College and Government General Hospital, Chennai. The study was reviewed and approved by the Institutional Ethical Committee. A total of 150 HIV positive patients presenting with clinical picture of oral candidiasis attending the ART center from June 2008-May 2009 were included in the study after getting informed written consent. Blood was collected in EDTA vacutainer tubes from each patients for enumeration of their CD4 count using BD Fluorescent Activated Cell Sorter (FACS) Counter.

Oropharyngeal specimens were collected by firmly swabbing the lesion site with two sterile cotton swabs under strict aseptic precautions. Specimens collected were subjected to standard mycological procedures. One swab was used for the direct microscopic examination by Gram stain and and observed for the presence of gram positive budding yeast cells with or without pseudohyphae. Second swab was inoculated immediately into the Sabourauds Dextrose Agar (SDA) plates supplemented with antibiotics and incubated at 28^0C and 37^0C for 48 -72 hours .Isolates were identified by colony morphology on SDA plates. The microscopic morphology of the colony on Gram stain was noted. Colonies that showed Gram positive budding yeast cells on Gram staining was further processed.

All the Candida isolates thus obtained were identified and speciated based on colony morphology, germ tube production, colony colour on HiCrom agar, chlamydospore production on corn meal agar (Dalmau plate technique), growth at 45°C, carbohydrate fermentation and carbohydrate assimilation pattern.

Antifungal susceptibility test for the Candida isolates was done by Microbroth dilution method, as per the guidelines of Clinical and Laboratory Standard Institutes (CLSI) on antifungal Susceptibility testing [14] . RPMI 1640 medium with glutamine, without bicarbonate in MOPS (3-N-

Morpholino propane sulfonic acid), buffer sterilized by membrane filtration was used as test medium.

The inoculum suspension was prepared by picking five colonies of 1mm diameter from a 24 hour old culture and suspended in 5ml sterile 0.85% NaCl. The turbidity of the cell suspension was adjusted to 85% transmittance at 530nm using a spectrophotometer by adding sterile 0.85% NaCl as necessary. The stock suspension (1×10^6 to 5×10^6 yeast cells/ml) prepared was further diluted in RPMI medium to achieve the final desired inoculums size containing 0.5×10^3 to 2.5×10^3/ml.

Serial twofold dilutions and final dilutions of each antifungal agent were prepared in RPMI 1640 medium. The Minimum Inhibitory Concentration (MIC) range of antifungal agents used were Fluconazole : 64 -0.125µg/ml and Itraconazole 16-0.031 µg/ml .Fluconazole was dissolved in sterile distilled water whereas itraconazole was dissolved in DMSO (Dimethyl sulfoxide). Aliquots of 100 µl of each antifungal agent at a concentration two times the targeted final concentration were dispensed in the wells of flat bottom 96-well microtiter plates . A constant volume (100 µl) of the inoculum was added to each microdilution well containing 100 µl of the serial dilution of the antifungal agents to reach final concentrations. The microtiter plates were incubated at 37°C for 48 hours.

The MICs of the fluconazole and itraconazole were defined as the lowest concentrations that inhibit growth by 50%. The MIC values for fluconazole and itraconazole were compared to the CLSI interpretative guidelines on antifungal susceptibility testing (Table-1). C. parapsilosis (ATCC 22019) and C. krusei (ATCC 6258) were used as controls for each test.

Statistical analysis were carried out using statistical package for Social sciences and Epi-software by statistician .The proportional data of this cross sectional study were tested using Pearson's Chi Square Analysis test and Binomial Proportion test.

RESULTS: In the present study, out of 150 patients with oral candidiasis, 102 (68%) were enrolled in this study during their first episode and the remaining 48 (32%) patients presented with recurrent candidiasis. Majority of the patients with recurrent candidiasis had a history of only one previous episode of oral candidiasis. Majority of the study population were within the age group of 29- 39 years with a male preponderance.

Candida was isolated from all the 150 patients (100%). As two patients harboured a mixture of two species, the total number of isolates were 152. Candida albicans was the most frequently isolated species 118 (77.6%) and the remaining were non-albicans species 34 (22.4%), with the frequency of C.tropicalis 17 (11.2%), C.krusei 8 (5.3%), C.parapsilosis 6 (3.9%), C.glabrata 2 (1.3%), C.guilliermondii 1 (0.7%). (Table-2)

Antifungal susceptibility testing of the Candida isolates revealed that about 121 isolates were susceptible, 13 were susceptible dose dependent, and 18 were resistant to fluconazole. One hundred and sixteen isolates were susceptible, 13 were susceptible dose dependent and 23 were resistant to itraconazole. (Table-3).

5.1% of the Candida albicans, 23.5% of the C.tropicalis, 87.5% of the C.krusei and 50% of the C.glabrata isolates showed MIC in the resistant range of >64µg/ml for fluconazole.10.2% of the Candida albicans, 35.3% of the C.tropicalis, 16.7% of C.parapsilosis, 37.% of the C.krusei and 50% of the Candida glabrata showed MIC in the resistant range of ≥ 1µg/ml for itraconazole (Table-4).

With reference to the immune status of the HIV patients with Candidiasis, 106 (70.7%) patients had CD4 count < 200 Cells/µl and the remaining 44 (29.3%) had CD4 count > 200 Cells/µl .There was a strong negative correlation between low CD4 count and oral candidiasis. (Table-5)

70.1% of the Candida albicans and 76.4% of the non albicans Candida sp. were isolated from the patients with CD4 count <200 cells / µl. Among the 34 non albicans isolates, 11 (32.2%) fluconazole resistant isolates were seen in patients with CD4 <200 cell/µl and 1 (2.9%) fluconazole resistant isolate was seen in patients with CD4>200 cells/ µl. Among the 118 Candida albicans isolates, 5 (4.1%) were seen in patients CD4 <200 cells/ µl and 1 (2.9%) fluconazole resistant isolate was seen in patients with CD4>200 cells/ µl. (Table-6)

DISCUSSION: Oral Candidiasis is the most common fungal infection in HIV infected patients and has been identified as a clinical predictor for progression to AIDS[15]. In the recent past, studies shows an increasing incidence of non-albicans Candida species. in Oral Candidiasis and increasing rates of antifungal drug resistance particularly with the immunocompromised patients. Rapid identification of candidiasis is important for the clinical management of immunocompromised patients.

In the current study, Candida was isolated from all the 150 patients. Various studies have shown the isolation rate of Candida ranging from 61-100 per cent in HIV infected individuals with oral candidiasis[16]. Species identification revealed that Candida albicans was the most frequently isolated species and among the non-albicans Candida spp, C.tropicalis was the most common species, a finding similar to other studies[17, 18]. C.dubliniensis was not isolated in this study. The evolving importance of non-albicans Candida spp.in HIV patients with oral candidiasis requires incorporation of standard techniques for Candida speciation.

Azoles are considered the drug of choice for treating oral candidiasis associated with HIV / AIDS patients[19]. Fluconazole is a triazole agent that has been widely used for the treatment of mucosal candidiasis because of its low toxicity and ease of administration[20].Several recent studies have reported fluconazole resistance in Candida strains isolated from HIV infected patients with oropharyngeal candidiasis. In the present study, 79.6% of the isolates were susceptible, 8.6% were SDD and 11.8% were resistant to fluconazole. This result is similar to that reported by other studies[17, 21, 22].

Itraconazole is used as an alternative to fluconazole for treating oral candidiasis.In this study 76.3% of the isolates were susceptible, 8.6% were SDD and 15.1% were resistant to itraconazole. C.tropicalis, C.glabrata and C.krusei accounted for more than half of azole resistance. Although C.krusei had a low prevalence, its intrinsic resistant activity against fluconazole may have therapeutic implications .Species other than C.albicans are generally less susceptible to therapy and arise mainly with low CD4 count and after repeated or prolonged antifungal treatment. This can be considered important since infections caused by C. albicans generally have the best prognosis in comparison to those caused by non-albicans species. C. glabrata and C. krusei remain the least susceptible species to fluconazole and because of cross resistance between azole drugs, they have high MICs to other azoles.[22, 23] The widespread use of fluconazole and itraconazole as therapeutic or prophylactic doses has increased recently and most often associated with the HIV infected with OPC. This has lead to the increase of reports of resistance[24].

Several authors have reported that apart from prolonged exposure, advanced immunosuppression is a major risk factor for azole resistance[16].In the present study, among the

150 HIV patients with oropharyngeal candidiasis, 70.7% had CD4 count <200 cells /mm^3 which was statistically significant by proportional test (p= <0.001) This correlated well with the study conducted by Usha et al[17] where 76.66% patients with oropharyngeal candidiasis had CD4 count <200 cells/ µl. Oral candidiasis can be used as a marker of immune status in field based settings where CD4 Count and viral RNA load estimation cannot be routinely done.

CONCLUSION: In conclusion, Candida albicans is the most frequently isolated species from HIV seropositive patients with oral Candidiasis. Non- albicans Candida species are emerging as important pathogens with increasing rates of azole resistance and with increased immunosuppression. The significant relationship of oral candidiasis with severe immunosuppression suggests that when oral candidiasis is present it can be used as a surrogate marker for CD4 depletion. Since oral candidiasis may serve as an early marker of immunosuppression in HIV patients, regular oral examination of these patients may help us to monitor the disease progression.. Further the increasing rates of resistance particularly among the non- albicans Candida spp. emphasizes the need for speciation and determination of antifungal susceptibility pattern of the oral candida isolates from HIV patients with oropharyngeal candidiasis.

	Susceptible	Susceptible Dose Dependent	Resistant
Fluconazole	≤ 8µg/ml	16-32 µg/ml	≥ 64 µg/ml
Itraconazole	≤ 0.125 µg/ml	0.25-0.5 µg/ml	≥ 1 µg/ml

TABLE -1: Interpretive Guidelines for In Vitro Susceptibility Testing of Candida species

Species	Total no	Percentage
Candida albicans	118	77.6
Candida tropicalis	17	11.2
Candida krusei	8	5.3
Candida parapsilosis	6	3.9
Candida glabrata	2	1.3
Candida guilliermondii	1	0.7
Total	152	100

TABLE -2: SPECIES DISTRIBUTION OF THE CANDIDA ISOLATES (n=152)

	Susceptible		SDD		Resistant	
	N	%	N	%	N	%
Fluconazole	121	79.6	13	8.6	18	11.8
Itraconazole	116	76.3	13	8.6	23	15.1

TABLE -3: ANTIFUNGAL SUSCEPTIBILITY BY MICROBROTH DILUTION METHOD

Species	No of isolates	Fluconazole by broth dilution			Itraconazole by broth dilution		
		Susceptible	SDD	Resistant	Susceptible	SDD	Resistant
		n	n	n	n	n	n
C.albicans	118	103	9	6	99	7	12
C.tropicalis	17	11	2	4	08	3	6
C.parapsilosis	6	6	-	-	5	-	1
C.Krusei	8	-	1	7	3	2	3
C.glabrata	2	-	1	1	-	1	1
C.guilliermondi	1	1	-	-	1	-	-
Total	152	121	13	18	116	13	23

TABLE-4: ANTIFUNGAL SUSCEPTIBILITY OF THE CANDIDA ISOLATES

CD4 Cells/ µl	No of Patients	Percent
<50	23	15.3
51-200	83	55.4
201-350	30	20
351-500	12	8
>500	2	1.3
Total	150	100

TABLE-5: CORRELATION OF ORAL CANDIDIASIS WITH THE CD4+ T CELL COUNT

CD4 cells/µl	Candida albicans (n=118)		Non albicans (n=34)	
	No of Resistant isolates n=6	Percentage of isolates resistant to fluconazole	No of Resistant isolates n=12	Percentage of isolates resistant to fluconazole
<50	03	2.5 %	02	05.8 %
51-200	02	1.6 %	09	26.4 %
201-350	01	0.8 %	01	02.9 %
351-500	-	-	-	-
>500	-	-	-	-

TABLE-6: CD4 DISTRIBUTION AMONG THE FLUCONAZOLE RESISTANT CANDIDA ISOLATES

REFERENCES:

1. Mary M Horgan, William G Powderly. Oral fungal infections. In: McGinnis MR, Anaissie EJ, Pfaller MA, editors. Pfaller's Clinical Mycology. Edinburgh: Churchill Livingstone, c2003, 1st ed:p446.
2. Benson CA, Kaplan JE, Masur H, Pau A, Holmes KK. MMWR Recommendations and Reports, Treating Oppurtunistic infections among HIV infected Adults and Adolescents. Recommendations from CDC.2004 Dec, 53 ; 1 -112.

3. Crowe SM, Carlin JB, Stewart KI, Lucas CR, Hoy JF. Predictive value of CD4 lymphocyte numbers for the development of opportunistic infections and malignancies in HIV infected persons. J Acquire Immune defici Syndr 1994, 4: 770 -776.
4. Dupont B, Denning DW, Marriot D, Sugar A, Vivani MA, Srisanthana T. Mycosis and AIDs patients. J Med Mycol. 1992, 32:19 -28.
5. Calvet HM, Yeaman MR, Filler SG.Reversible fluconazole resistance in Candida albicans: a potential in vitro model. Antimicrob Agents Chemother. 1997, 41: 535 -539.
6. Anwar KP, Malik A, Subhan KH. Profile of candidiasis in HIV infected patients. Iranian journal of Microbiology. 2012 Dec;4 (4):204-208.
7. Brown ER, Dresser LD, Chin WF. Superficial fungal infections. In: Dipiro JT, Robert LT, Yee GC, Matzke GR, Wells BG, Possey LM, editors. Pharmacotherapy, a pathophysiologic approach. 8th edition:2055 -2073
8. Mousavi SAA, Salari S, Rezaic S, Nejad NS, Hadizadeh S, Kamyabi H, Aghari H. Identification of Candida Species Isolated From Oral Colonization in Iranian HIV-Positive Patients, by PCR-RFLP method. Jundhishapur J Microbiol 2012, , 5 (1):336 - 340.
9. Juergen Loeffler and David A Steven. Antifungal drug resistance, Clin Infect Dis 2003, Vol 36 (supplement 1);S31-S41.
10. Maertens JA: History of the development of azole derivatives. Clin Microbiol Infect 2004, 10 (Supp 1): 1- 10
11. Colombo AL, Da Matta D, De Almeida LP, Rosas R: Fluconazole susceptibility of Brazilian Candida isolates assessed by a disk diffusion method. J Infect Dis 2002, 6:118-123.
12. Mulu A, Kassu A, Anagaw B, Moges B, Gelaw A, Alemayehu M, Belyhun Y, Biadglegne F, Hurissa Z, Moges F and Isogai E. Frequent detection of azole resistant Candida species among late presenting AIDS patients in northwest Ethiopia; BMC Infectious Diseases 2013, 13:82.
13. Coppola S, Angarano G, Montagna MT, Congedo P, Monno L, Bellisario A, Pastore G.Efficacy of itraconazole in treating AIDS associated infections due to Candida krusei. Europ. J. Epidemiolog 1997. 11:243-244.
14. Clinical and Laboratory Standards Institute (CLSI). Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard M27-A2, 2nd edition. Wayne, PA: Clinical Laboratory Standards Institute; 2002.
15. Vargas KG, Joly S. Carriage frequency, intensity of carriage, and strains of oral yeast species vary in the progression to oral candidiasis in human immune deficiency virus - positive individuals. J Clin Microbiol 2002; 4: 341-50.
16. Mane A, Panchvalli S, Bembalkar S and Risbud A. Oral Candida colonization in HIV infected individuals. Indian J Med Res 131, June 2010: 836-838.
17. CA Enwuru et al. Fluconazole resistant oppurtunistic oesophageal Candida and non- Candida yeast like isolates from HIV infected patients attending ARV clinics in Lagos, Nigeria, African Health sciences, Sep:2008; 8 (3);142-148.
18. Arora U, Jagdev M, Jindal N. Immunosuppression level in HIV-positive patients with oropharyngeal Candidiasis. Indian J Med Microbiol 2009; 27: 174-5.
19. White TC, Hollemann S, Dy F, Mirels LF, Stevens DA. Resistance mechanisms in clinical isolates of Candida albicans. Antimicrobial Agents Chemother, 2002; 46; 1704 -1713.

20. Badiee P, Alborzi A, Davarpanah MA, Shakiba E. Distributions and Antifungal Susceptibility of Candida Species from Mucosal Sites in HIV Positive Patients. Archives of Iranian Medicine.2010 Jul;13 (4):282-7.
21. Hamza OJM, Matee MIN, Moshi MJ, Simon ENM, Mugusi F, Mikx FHM, Helderman WH, Rijs AJ, Van der Ven AJ, Verweij PE.Species distribution and in vitro antifungal susceptibility of oral yeast isolates from Tanzanian HIV infected patients with primary and recurrent oropharyngeal candidiasis. BMC Microbiol. 2008. 8: 135.
22. Costa CR, De Lemos JA, Passos XS, de Araújo CR, Cohen AJ, Souza LK, Silva Mdo R. Species distribution and antifungal susceptibility profile of oral Candida isolates from HIV-infected patients in the antiretroviral therapy era. Mycopathologia.2006 Jul; 162 (1): 45-50.
23. Mokaddas EM, Al-Sweih NA, Khan ZU. Species distribution and antifungal susceptibility of Candida bloodstream isolates in Kuwait: a 10-year study. J Med Microb. 2007; 56**:** 255 – 259.
24. Meurman JH, Siikala E, Richardson M, Rautemaa R. Non Candida albicans Candida yeasts of the oral cavity. Communicating Current Research and Education Topics and Trends in Applied Microbiology. 2007:719-731.

AUTHORS:

1. Kalpana Devi V.
2. Geetha Lakshmi S.

PARTICULARS OF CONTRIBUTORS:

1. Assistant Professor, Department of Microbiology, A.C.S. Medical College & Hospital, Velappanchavadi, Chennai.
2. Dean, Professor, Department of Microbiology, Stanley Medical College and Hospital, Chennai.

NAME ADRRESS EMAIL ID OF THE CORRESPONDING AUTHOR:

Dr. Kalpana Devi V,
No. 5/75, Kasi Avenue,
R.R. Nagar, 3rd Street Annexe,
Ayyapanthangal, Chennai – 600 056.
Email – drkalpana2k@yahoo.co.in

Date of Submission: 14/11/2013.
Date of Peer Review: 15/11/2013.
Date of Acceptance: 20/11/2013.
Date of Publishing: 23/11/2013