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International Journal of Current Research
Vol. 7, Issue, 06, pp.16996-17000, June, 2015

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

A STUDY OF ORAL CANDIDAL CARRIAGE AND CD4 T-LYMPHOCYTE COUNT IN HIV POSITIVE PATIENTS AND ANTIFUNGAL SUSCEPTIBILITY OF ISOLATED CANDIDAL STRAINS FOR FLUCONAZOLE AND VORICONAZOLE IN VITRO

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ARTICLE INFO

Article History:
Received 10th March, 2015
Received in revised form
17th April, 2015
Accepted 05th May, 2015
Published online 27th June, 2015

Key words:

Antifungal Susceptibility,
CD4 T-Lymphocytes; HIV,
Oral Candidal Carriage.

ABSTRACT

Objectives: To detect the prevalence of asymptomatic Candidal colonization among HIV positive and Healthy individuals and to investigate the relationship of asymptomatic Candidal colonization of oral cavity and CD4 T-lymphocyte count and to assess antifungal drug susceptibility for Candida species by Fluconazole and Voriconazole in vitro.

Methods: Asymptomatic candidal Colonization of oral cavity were investigated in total 50 HIV positive patients having CD4T- lymphocyte count < 500 cells / cu. mm and absence of antifungal treatment at Acharya Vinoba Bhave Rural Hospital Wardha, India. Fifty healthy controls were selected to ensure comparability. Oral swabs were collected from tongue and palate, Candidal species isolated and antifungal susceptibility testing had conducted based on disk diffusion procedures.

Results: Prevalence of Candidal colonization was 52% among the cases which was significantly higher than the healthy control(8%) (p=0.00001). There is no association between asymptomatic Candidal colonization on tongue and palate with decreasing CD4 T lymphocyte count statistically (P=0.3454, P=0.7279). Antifungal susceptibility, among cases, Candidal colonization, 78.8% and 71.1% were sensitive and 17.3% and 26.9 % were resistant to Fluconazole and Voriconazole respectively.

Conclusions: High prevalence of Candidal colonization found among HIV positive individuals. Asymptomatic oral Candidal colonization is not related to CD4 T- lymphocyte count. Antifungal drug sensitivity must be done.

***Citation*:** Monal, M. Kukde, Deepak, S. Selokar, Rahul, R. Bhowate, Silpi Basak and Shirish, S. Degwekar, 2015. "A study of oral candidal carriage and cd4 t-lymphocyte count in hiv positive patients and antifungal susceptibility of isolated candidal strains for fluconazole and voriconazole in vitro", *International Journal of Current Research,* 7, (6), 16996-17000.

INTRODUCTION

Oral Candidiasis is the most common in Human Immunodeficiency Virus (HIV)-related oral lesion. The presence of oral lesions may be an early diagnostic indicator of immunodeficiency and HIV infection and is a predictor of progression of HIV disease (Lifson, 1994; Katz, 1992). The principal effect of HIV on the immune system is the depletion of CD4 T lymphocytes with advancing disease (McCarthy, 1992). Treatment of oropharyngeal candidiasis involves the use of topical or systemic antifungal therapy.

Unfortunately because of the profound and sustained immunosupression in AIDS patients, relapse or reinfections are common. For these reasons, patients with AIDS commonly receive antifungal therapy for multiple oral infections with C.albicans over an extended period of time which leads to the development of resistance among isolates of Candida species.

Hence a need was felt to define the prevalence of asymptomatic Candidal colonization in the oral cavity of HIV infected individuals and to find out relationship of oral candidiasis with CD4 T lymphocyte count and also to evaluate the efficacy of antifungal drugs on isolated Candidal strains in vitro in HIV positive individuals.

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MATERIALS AND METHODS

The present Case control study was conducted during 2008-2010, among 50 HIV positive patients as cases who were admitted in the community care centre of Acharya Vinoba Bhave Rural Hospital, Wardha, India. Institutional Ethical Committee (IRB) of Datta Meghe Institute of Medical Sciences, University, and Wardha had approved the protocol of the study [DMIMSU/IEC/2008-2009/44. Date: 29/11/2008]. The Inclusion criteria for cases were : (i) Patients who has been diagnosed as HIV seropositive at Integrated Counseling and Testing Centre (ICTC) among age group ≥18yr; (ii) Patients having CD4 lymphocyte count < 500 cells / cu. mm ; (iii) Absence of antifungal treatment within last 3 months. Patients with xerostomia and salivary gland disease, pregnant and nursing woman were excluded from study. Fifty healthy individuals as controls were selected who were health care workers in Sharad Pawar Dental College and Jawaharlal Nehru Medical College, Wardha to ensure comparability. The Health care workers selected who having age > 18 years , who did not have high risk exposure and were physically healthy with normal oral mucosa and had not received antifungal treatment within last 3 months.

Data collection

A structured proforma was used to collect information just before collecting the specimen. Written informed consent has been taken from each subject. Confidentiality was maintained. Socio-demographic information like age, sex, occupation, monthly income, education & marital status was recorded. Extra oral examination was done which includes examination of regional group of lymph nodes. Intra oral examination includes examination of lips, buccal mucosa, tongue, hard palate & soft palate for any association of lesions. Information including date of HIV antibody testing as per National AIDS Control Organization (NACO) guidelines and CD4 count with date and duration of taking ART was noted.

Microbiological Procedures

From each patient two oral swabs were collected from posterior hard palate and tongue. With one swab Gram staining was done for budding yeast cells with pseuduhyphae and the other swab was inoculated on Sabouraud Dextrose Agar (M1067500) with Chloramphenicol and plates were incubated at 37^0 for18-20 hrs. and are white to cream colour, smooth and glistening colonies were observed. The direct microscopic examination of clinical specimens containing Candida reveals budding yeast cells (blastoconidia) approximately 2-4 micrometer in diameter and /or pseuduhyphae showing regular points of constriction, resembling links of sausage. Subsequently, it was subcultured on Hi Chrome Candida differential Agar (M1297A-100G) in order to differentiate species of Candida. After CHRO Magar light green colour colonies for Candida albicans were further confirmed by rapid identification by germ tube (GT) test. Antifungal susceptibility testing had conducted primarily based on disk diffusion procedures as per CLSI guidelines of antifungal drugs (Approved guideline M-44A, CLSI, USA).

Antifungal susceptibility test

Antifungal susceptibility test were done by antifungal disk diffusion susceptibility testing of yeasts as per CLSI guidelines M-44A, 2006 for Fluconazole and Voriconazole. The inoculums of the test strains of Candida was prepared in sterile saline with turbidity adjusted to 0.5 McFarland Lawn culture of the test strain as then done on Muller –Hinton agar (M1084-500G) plate, plate supplemented with 2 % glucose and 0.5 µ/ml. Methylene blue dye (pH 7.2 to 7.6). Antifungal disks containing fluconazole (FU^{25} 25 mcg/disc) and Voriconazole (VOR^1 1mcg/disc) were put evenly in order to avoid intermingling so that they are not closer 24 mm from centre to centre. Then the plates were incubated at 35^0 C for 24 hours. The zone sizes were measured as susceptible, resistant and susceptible –dose dependent.

Interpretation

Antifungal Agents	Disc contents	Zone diameter,(Nearest whole in mm)		
		Resistant	Susceptible dose dependent	Sensitive
Fluconazole	25µg	≤ 14	15-18	≥ 19

Quality control culture of C.albicans (ATCC 90028) and C.krusei (ATCC 6258) for the reproducibility and accuracy of disk diffusion test.

Data Analysis

Statistical analysis of data was analyzed by using Microsoft Excel and Graph Pad Instat3 statistical software. Data was tabulated using frequency distribution tables. Frequency of demographic characteristics, various clinical and laboratory findings were expressed as proportions (%). Mean and Standard deviation were used for quantitative data. Association between asymptomatic oral Candidal colonization and CD4 count and difference of Prevalence of asymptomatic Oral Candidal colonization among cases and controls was determined using Chi-square test for linear trend. The level of significance was taken at P value <0.05. Odds ratio and confidence interval were also calculated. For confounding variable adjusted Odds Ratio has been calculated using Mantel-Haenszel test.

RESULTS

In the current study, among 50 cases 26 (52%) were males 24 (48%) were females. The age ranged from 26 -68 years with the mean age and SD being 38.4 ± 9.95. The age of total 50 Controls in the study ranged from 22-58 years with the mean age and SD being 33.5 ± 9.10. Out of 50 controls 30 (60 %) were males and 20(40%) were females. Regarding educational status of cases, 8 (16%) cases were illiterate, 42(84%) literate. However 40 % of cases were labourer by occupation. Among the cases; 25(50%) were tobacco chewers, 4(8%) were smokers and 3(6%) of them were addicted to tobacco and chewing and smoking. 18 (36%) were did not have any adverse habits. Among controls, 35 (70 %) were did not have any adverse habits as shown in Table 1. The number of specimens (cases) which shows growth of Candida on tongue was 31 while on palate was 21 and the control group it is 5 and 3 respectively. The asymptomatic candidal carrier rate was 52 % (52 out 100) among cases which was higher than the controls (8%) and statistical significant difference was

observed (Chi-square test, p=0.00001) as given in Table 2. As some cases and controls has one or more type of tobacco related habits, to remove the effect of this confounding factor on candidal growth adjusted Odds ratio has been calculated after applying Mantel-Haenszel test.

species among cases were sensitive to Fluconazole and Voriconazole respectively while 9(17.3%) and 14(26.9%) were resistant to fluconazole and Voriconazole respectively and 2(3.8%) and 1(1.9%) were susceptible dose dependant to fluconazole and Voriconazole respectively as shown in Table 4.

Table 1. Demographic Profile and Habits of cases and controls

		HIV + Individuals (n=50)	HIV – Healthy Individuals (n=50)
Sex	Male	26 (52 %)	30 (60%)
	Female	24 (48%)	20 (40%)
Age	Mean Age	38.4	33.5
	SD	±9.95	± 9.10
Education	Literate	42 (84 %)	50 (100 %)
	Illiterate	8 (16 %)	0
Occupation	Laborer	20 (40 %)	50 (100%) All are Health Care workers
	Others(Bussiness,Farmer, Housewives, Service)	30 (60 %)	
Habits	Tobacco Chewer	25 (50%)	10 (20%)
	Smoker	4(8%)	4 (8%)
	Both	3 (6%)	1 (2%)
	No habbit	18 (36%)	35 (70%)

Table 2. Comparison of Candidal growth on tongue and palate among Cases and Controls

Specimens showing Growth of Candida		No. of specimens of Cases (n=100)	No. of specimens of control (n=100)	X^2 test P value	Odds Ratio	95 % Confidence Interval
On Tongue	Yes	31	5	p=0.00001	12.43	4.214-36.653
	No	19	45			
On Palate	Yes	21	3	p=0.00001	10.44	2.854-38.223
	No	29	47			

Table 3. Association between CD4 count and Candidal growth on tongue and palate

CD4 count (cells /cu.mm)	Growth of candida on tongue (n=50 specimens)		X^2 test for linear trend; P value	Growth of candida on palate (n=50 specimens)		X^2 test for linear trend; P value
	Yes	No		Yes	No	
301-499	7	6	P=0.3454	5	8	P=0.7279
101-300	16	10		11	15	
< 100	8	3		5	6	
Total	31	19		21	29	

Table 4. Antifungal susceptibility for Candidal colonization isolated from tongue and palate of Cases and Control

Number of Candidal colonization		Fluconazole			Voriconazole		
		Sensitive (%)	Resistant (%)	SDD* (%)	Sensitive (%)	Resistant (%)	SDD* (%)
Cases	Tongue(n=31)	26(83.9)	4(12.9)	1(3.2)	22(70.9)	8(25.8)	1(3.2)
(n=52)	Palate(n=21)	15(71.4)	5(23.80)	1(4.8)	15(71.4)	6(28.6)	0
Control	Tongue(n=5)	3(60)	2(40)	0	3(60)	1(20)	1(20)
(n=8)	Palate(n=3)	2(66.6)	1(33.3)	0	1(33.3)	2(66.6)	0

* SDD- Susceptible dose dependant

The adjusted Odds Ratio for growth of candida on tongue is 14.45 and on palate is 8.60. The association between CD4 count and candidal growth on tongue and palate in HIV positive patients is shown in Table 3. Among 100 specimens (cases) which show growth of Candida on tongue was 31 while on palate was 21. Thirteen Specimens shows growth of candida from tongue and palate were at CD4 + lymphocyte count < 100 cells/ cu.mm and fifty two specimens were at CD4 count 101-300 cells/ cu.mm, 27 shows growth of Candida. However 26 specimens were at status of CD4 count 301-499 cells/ cu.mm, 12 shows growth of Candida. In the present study, there is no trend of increasing candidal growth with decreasing CD4 count (P=0.3454, P=0.7279).Among cases C.albicans was found as the most common isolated species which was followed by C.glabrata, C.tropicalis, C.dubliensis and C.krusie. Forty one (78.8%) and 37(71.1%) candida

DISCUSSION

Yeasts are often isolated from the oral cavity of both healthy individuals as well as hospitalized patients. The number of factors which includes immunity of host, the strain of candida, oral hyegine status, smoking, prior use of antibiotics and general health of host will affect the asymptomatic Candidal carrier state. Due to various collection methods and different background of subjects prevalence of asymptomatic oral Candidal carriage in earlier studies are different (X liu *et al.,* 2006). For instance, some authors isolated yeasts in 66.66 % of AIDS group, 10.81 % of the HIV-free healthy group, and 57.14% of the HIV- free candidiasis group (Teanpaisan *et al.,* 1998). Also a yeast positivity rate was found 63.5 % among HIV positive individuals (Schoofs *et al.,* 1998) while 61.9 % in the HIV positive group and 29.3 % in HIV negative group

(Campisi *et al.,* 2002). In another study authors found that oral Candida carriage rate in HIV positive patients (28.6 %) was a little higher than that in the healthy group (18.0 %) (X liu *et al*., 2006). Likewise our study revealed that the Oral Candidal carriage rate among HIV positive individuals (52 %) was significantly higher than that in healthy group (8%) (p=0.0001). The asymptomatic oral candidal carriage rate was found to be higher among HIV positive subjects which may be due to poor oral hygiene status and majority of subjects were labourer who belongs to poor socioeconomic strata.

The association between oral Candidiasis and CD4 T-lymphocyte count among HIV positive individual is important as it can be used as indicator of immune status in absence of availability of CD4 T-lymphocyte testing. In the present study , the specimens of HIV positive patients with CD4 –T Lymphocyte count less than 100 / cu.mm had more yeast colonization i.e. 13/22 (59 %) than the patients having CD4 count 101-300 /cu.mm is 27/52 (52 %) and the patients having CD4 count 301-499 cells/ cu.mm 12/26 (46 %). Although decrease in CD4- T lymphocyte count in HIV infected individuals showed more candidal colonization but this difference was statistically non significant (p=0.3454; p=0.7279). In a study, a yeast carriage rate in HIV positive individual were found 67% for those with CD4 cell counts above 500 cells/cu.mm , 86 % for patients with counts of 200 to 500 cells /cu.mm and 82 % for patients with CD4 cell counts below 200 cells / cu.mm (Fong *et al.,* 1997). In another study , authors noted that Candida infection of oropharyngeal mucosa was associated with highly significant reductions in CD4 lymphocyte counts and esophageal candidiasis occurred only with advanced immunodeficiency associated with CD4 counts less than 100 / cu.mm (Imam *et al.,* 1990). In consistent with our study some authors found that asymptomatic oral Candidal colonization is not related to CD4 T-lymphocyte count in individuals with HIV infections (X liu *et al*., 2006). Although previous studies as well as our study suggest no statistical significant association between CD4 T-Lymphocyte count and asymptomatic oral Candidal colonization, still candidal carriage rate is higher amongst HIV positive individuals. Hence thorough examination of the oral cavity and CD4 T-Lymphocyte count should be done which is beneficial for health and well being of people living with HIV disease.

Fungal infections are caused due to eukaryotic organisms which results more difficult therapeutic problems than do bacterial infections. During the initial episodes of oral Candidiasis topical antifungal treatment is useful, but most patients suffer multiple episodes and fluconazole or itraconazole can help in the management. Voriconazole may be used for recalcitrant oral and oesophageal candidiasis as a alternative agent. Antifungal susceptibility testing should be done by standardized testing methods such as those from CLSI or EUCAST which are time consuming but have improved the reproducibility of testing results. Regarding antifungal susceptibility, we reported mixture of susceptible and resistant candidal strains among cases; 41(78.8%) and 37(71.1%) were sensitive to Fluconazole and Voriconazole respectively while 9(17.3%) and 14(26.9%) were resistant to fluconazole and Voriconazole respectively and 2(3.8%) and 1(1.9%) were susceptible dose dependant to fluconazole and Voriconazole respectively. For instance, some author found that Fluconazole resistance was detected in 3.2 % of all candidal isolates and 98 % were considered susceptible to Voriconazole (Thean Yen Tan *et al*., 2008). Furthermore Vargas LOS *et al.* (2010) found that the in vitro susceptibility of oral yeast isolates for fluconazole and variconazole were 95.7 % and 100 % respectively. In another study, Troillet *et al.* (1993) stated that an advanced stage of HIV infection and previous exposure to fluconazole could be risk factors for the development of fluconazole resistant oropharyngeal candidiasis. In contrast to above studies of antifungal susceptibility, Drobacheff *et al.* (1996) demonstrated that prolong treatment with fluconazole dose higher than 13 gm induces the emergence of resistant of C.albicans (33%) with persistence of the same C.albicans strain and these findings are similar to our study. The development of resistance of Candidal strains to azoles has been associated with prior exposure to irrational use of antifungal drugs mainly azoles, long standing HIV infection and severe immunosuppression states. In the view of emergence of resistance our study suggested that choose carefully antifungal drugs for fungal infections in patients with AIDS after doing in vitro antifungal susceptibility.

In conclusion, high prevalence of Candidal colonization found among HIV positive individuals among which C.albicans is the most common recovered species. The association of asymptomatic oral Candidal colonization is statistically not related to CD4 T-lymphocyte count. Most of the Candida isolated from HIV positive individuals is more sensitive to Fluconazole as compared to Voriconazole. Due to rising emergence of resistance to azoles, these agents should be used cautiously and should be preserved for severe candidiasis or clinically symptomatic patients.

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