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Original Research Article

Speciation of *Candida* and antifungal susceptibility from oral thrush cases in people living with HIV and its correlation with CD4 count and viral load in a tertiary care hospital: a cross-sectional study

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ABSTRACT

Background: Oropharyngeal candidiasis (OPC) is the most common opportunistic infection observed in Human immunodeficiency virus (HIV) seropositive patients. It has been perceived that low CD4 counts and high plasma HIV RNA levels, both significantly correlate with oral candidiasis in HIV patients. Aim was to determine the antifungal susceptibility testing of the *Candida* isolates by disc diffusion method.
Methods: The present study is a cross-sectional study that included 100 clinical samples processed for speciation of *Candida* isolates by using standard mycological techniques and antifungal susceptibility of the *Candida* isolates was done by using disc diffusion method and minimum inhibitory concentration (MIC's) was done by 'E' strip test for fluconazole resistant isolates. CD4 count was estimated by using flow cytometry method and viral load was estimated by using real time PCR, Abbott M2000SP and correlated with oral thrush.
Results: Out of 100 *Candida* isolates, *C. albicans* 84 (84%) was the predominant species followed by *C. krusei* 7 (7%), *C.* glabrata 6 (6%), *C. tropicalis* 3 (3%). Among the antifungals used in this study, the most sensitive agent was voriconazole 99 (94.4%) and the least was itraconazole 59 (19.6%). CD4 count was less than 200 cells/µl in 45 (45%). Viral load varied from 1,62409 to 58 copies/ml of blood in the patients with oral thrush with significant association.
Conclusions: To conclude, though *C. 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.

Keywords: Oral candidiasis, *Candida* species, Antifungal susceptibility testing, CD4 count, Viral load

INTRODUCTION

Human immunodeficiency virus (HIV) related opportunistic fungal infections (OFIs) cause of morbidity and mortality. It has been estimated that 60% to 90% of people with HIV disease will present with at least one oral manifestation.[1] The importance of oral lesions as clinical biomarker indicator of HIV infection and predictor of progression of HIV to AIDS. OPC is the most common opportunistic infection observed in HIV seropositive patients, occurring in an estimated 80 to 95% of these patients when the CD4 T lymphocyte counts are below 200 cells/mm is an early indication to indicate that immune system is impaired in HIV-infected patients. The low CD4 counts and high plasma HIV RNA levels, both significantly correlate with oral candidiasis in HIV

patients. These observations suggest that decreasing the viral load and increasing the CD4 count by initiation of highly active antiretroviral therapy (HAART) would reduce the need for specific antifungal therapy. Increased retroviral replication and an associated decline in immune defences render these patients more susceptible to oropharyngeal candidiasis.[2,3] 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.[4] OPC is considered as one of the earliest indicators 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.[5] *C. 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.[6] Antifungal drug resistance is fast becoming a major problem. The high incidence of mucosal and deep-seated forms of candidiasis has resulted in the use of systemic antifungal agents, especially fluconazole and itraconazole.[7] Many of these patients require long-term treatment to suppress oropharyngeal candidiasis. The widespread use of these antifungal agents has been followed by an increase in antifungal resistance and by a noticeable shift toward non albicans species with relative resistance to fluconazole and itraconazole and there have been reports of emergence of resistance to antifungal agents in HIV/AIDS patients with OPC.[8,9] The increased reports of antifungal resistance and expanding drug therapy options prompted the need for clinically relevant antifungal susceptibility testing. Prompt use of antifungal drugs judiciously reduces the advent antifungal resistance. Further the emergence of other *Candida* species such as *C. krusei* and *C. glabrata* with innately reduced susceptibilities to fluconazole also results in treatment failure, emphasizing the need for speciation of the oral yeast isolates.[10]

The aim of the present study is identification of *Candida* species from the clinical isolates and detection of antifungal susceptibility testing by Kirby Bauer disc diffusion method.

The primary objective is the detection of MIC's using the E strip test. The secondary objective is the correlation of CD count by flow cytometry and viral load.

METHODS

The present study was a cross-sectional study conducted in the department of microbiology, Rangaraya medical college, Kakinada, Andhra Pradesh, India, for a period of one year from September 22, 2023 to September 23, 2024. Ethics committee approval (IEC/RMC/2023/1055) for the study was obtained before initiating the study. Informed consent was obtained from each patient before sample collection. Blood was collected in EDTA vacutainer tubes from each patient for enumeration of their CD4 count using flow cytometry method. Oropharyngeal specimens were collected by firmly swabbing the lesion site with two sterile cotton swabs under strict aseptic precautions, taking care not to contaminate the swab with saliva.

Inclusion criteria

Seropositive patients, patients of age group 20-60 years, patients willing to give an informed consent were included.

Exclusion criteria

Seronegative patients, patients who were on antifungals for the last 6 months, and the patients who are not willing to give an informed consent were excluded.

Study procedure

Specimen collection

Oral cavity of the patients was examined for white plaques signs of candidiasis. Two sterile swabs were collected from the site of the lesion under strict aseptic precautions. One swab was used for the direct smear by Gram stain and the other for culture. Specimens collected were subjected to standard mycological procedures. Five milliliter of whole blood was collected from the patient and blood was drawn into EDTA anti-coagulated bottle for CD4 count estimation.

CD4 cell count estimation

The CD4 count T lymphocyte count of all the patients was determined by the Sysmex flow cytometer. The instrument works on the principle of flow cytometry.

Microscopy

The direct smear shows pus cells with gram positive budding yeast cells with/without pseudo hyphae (shown in the Figure 1) Gram stain of an oropharyngeal swab was a valuable initial diagnostic tool to suspect the presence of *Candida*.

Culture

The other swab was inoculated onto Sabouraud's dextrose Agar, incubated at 37°C for 24-48 hours and observed for cream coloured, smooth, pasty colonies. Yeast like colonies were subjected to Gram stain for confirmation (Figure 2).

Speciation of Candida

Candida isolates 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 test.

A small proportion of isolated colony suspended in test tube containing 0.5 ml of human serum. The test tube was incubated at 35ºc for not more than two hours. A drop of yeast-serum suspension was taken on microscope slide overlaid with coverslip and examined under 40× objective lens for presence or absence of germtubes. Germ tubes were positive for *C. albicans* and *C. dubliensis,* both were differentiated by growth at 45ºc by *C. albicans* whereas *C. dubliensis* does not grow at 45º C.

A heavy inoculum of yeast was streakd across plate containing cornmeal agar with tween 80 and coverslip as placed over it. The streak should project beyond cover slip. Plates were incubated at 25º C for 24-48 hours. Examination was done for presence of chlamydospores, arrangement of pseudohyphae and observed under the microscope 40× objective.

Hichrom Candida agar

Streaking was done on Hichrom *Candida* agar with an isolated colony of yeast. Colour and morphology of the colony was observed after 48 hours of incubation. Light green colour *C. albicans,* light pink colour *C. krusei,* cream colour *C. glabrata* and steel blue colour *C. tropicalis* were seen (Figure 3).

Carbohydrate fermentation test

Sugar fermentation test was done to detect acid and gas production from 2% glucose, maltose, sucrose, lactose, trehalose and galactose with Andrade's indicator and Durham's tube, incubated for 5-7 days at 25ºC. Pink colour in the test tube indicates acid production and bubble in Durham's tube indicates gas production (shown in the Table 1).

Antifungal susceptibility testing

Antifungal susceptibility testing was done by disc diffusion method as per CLSI guidelines M44-A. The anti-fungal drugs tested were fluconazole 25 µg, itraconazole 10 µg, voriconazole 1 µg, caspofungin 5 µg, amphotericin 100 units. A total 100 *Candida* isolates; antifungal susceptibility was done by disc diffusion method.

Out of 100 *Candida* isolates, 18 isolates were fluconazole resistant which were subjected to MIC's. Inoculum is prepared by picking five distinct colonies of about 1 mm in diameter from a 24- hour-old culture of *Candida* species.

Colonies are suspended in 5 ml of sterile 0.145 mol/L saline (8.5g/L NaCl; 0.85% saline). Suspension is vortexed for 15 seconds and its turbidity is matched to 0.5 McFarland standard. Lawn culture was made using a sterile cotton swab in three directions on Muller Hinton Agar plate supplemented with 2% glucose and 0.5 µg/ml methylene blue, discs were placed on the surface of agar and incubated at 37ºC for 24 hours.

The diameter of zone of inhibition was measured and compared with standard zones interpretive breakpoints according to CLSI M44-A2.

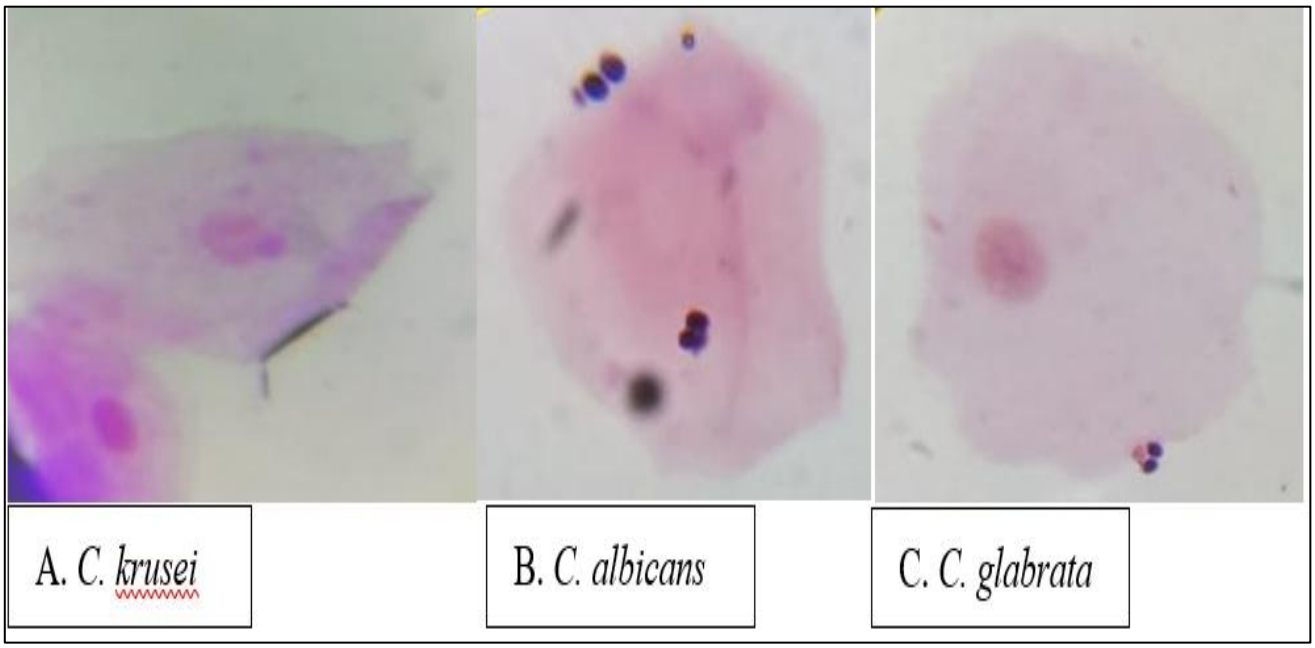

Figure 1 (A-C): Direct smear-microscopy of Gram stain under 100× objective.

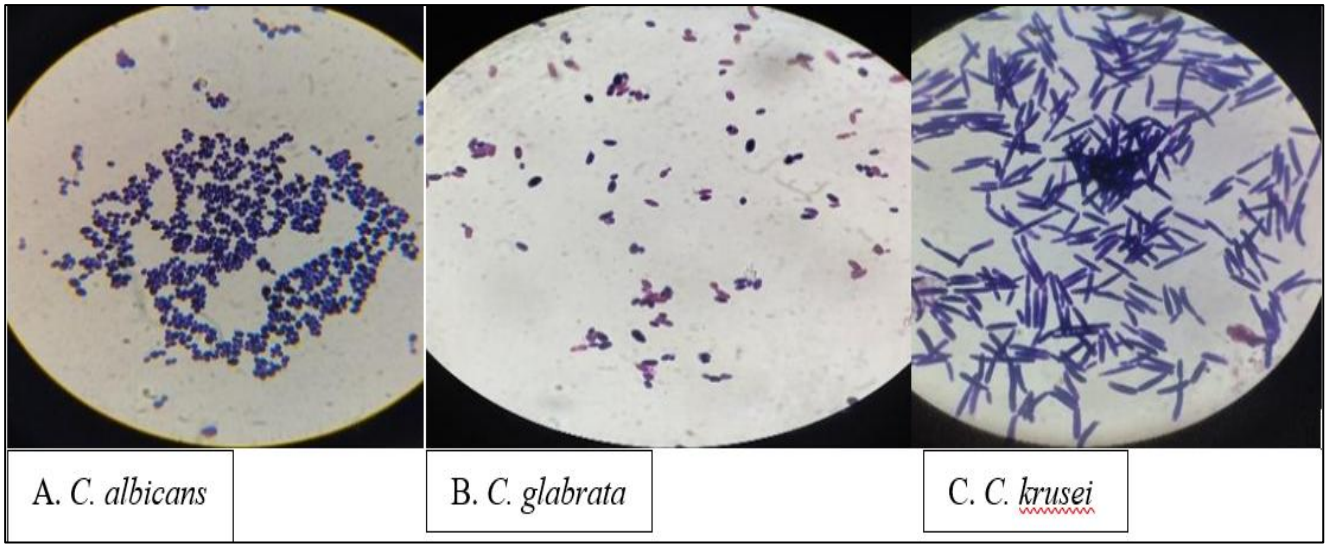

Figure 2 (A-C): Microscopic examination of yeasts on culture under 100× objective.

Figure 3: Hichrom *Candida* agar.

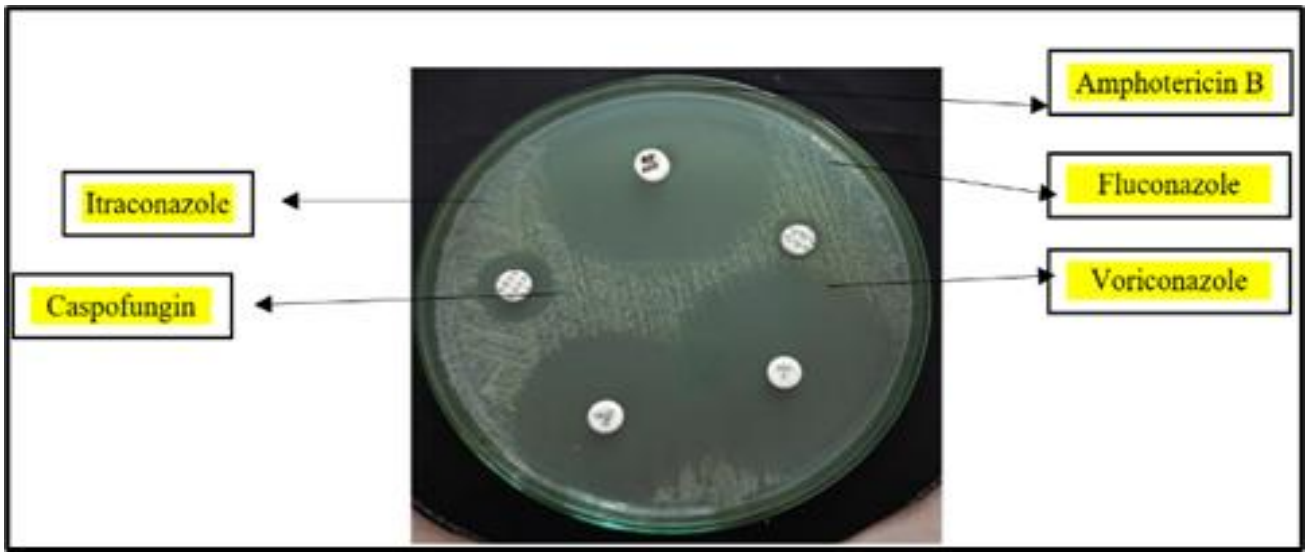

Figure 4: Antifungal susceptibility testing by disc diffusion method.

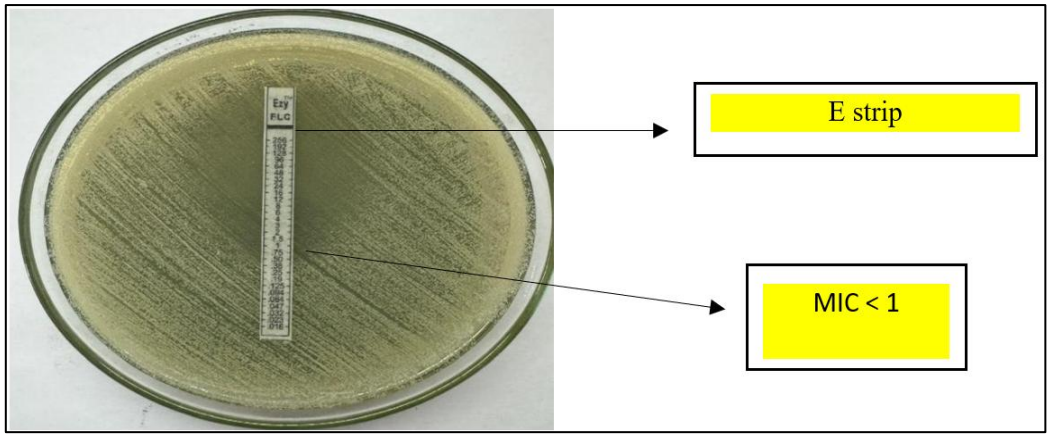

Figure 5: Antifungal susceptibility testing by E test.

Table 1: Speciation of *Candida* by sugar fermentation test.

Candida species	N	Fermentation of sugars					
		Glucose	Lactose	Sucrose	Maltose	Trehalose	Galactose
C. albicans	84	Acid with gas	-	-	AG	AG	AG
C. krusei	7	AG	-	-	-	-	-
C. glabrata	6	AG	-	-	-	AG	-
C. tropicalis	3	AG	-	AG	AG	AG	AG

*Note: AG indicates acid and gas; Indicates no acid and gas.

Table 2: Speciation of *Candida* by sugar assimilation test.

Candida species	N	Assimilation of sugars					
		Glucose	Lactose	Sucrose	Maltose	Trehalose	Xylose
C. albicans	84	Assimilates	-	Assimilates	Assimilates	Assimilates	Assimilates
C. krusei	7	+	-	-	-	-	-
C. glabrata	6	+	-	-	-	+	-
C. tropicalis	3	+	-	+	+	+	+

*Note: + indicates sugar assimilates; -indicates sugar not assimilates

Statistical analysis

Data collected was entered in Microsoft excel sheet. Tables with frequency and percentages are calculated.

RESULTS

A total of 100 patients were included in this study with 34 (34%) females and 66 (66%) males. The minimum and the maximum age of the patients were 22 and 65, respectively. *C. albicans* was the most frequently isolated species 84 (84%) and the remaining were non-albicans species 16 (16%), with the frequency of *C. krusei* 7 (7%), *C. glabrata* 6 (6%), *C. tropicalis* 3 (3%).

Antifungal susceptibility testing of the *Candida* species, out of 84 isolates of *C. albicans* showing sensitive to fluconazole 69 (82.1%), SDD 15 (17.8%) resistant 3 (3.5%), for itraconazole, sensitive 54 (64.2%) SDD 4 (4.7%), resistant 3 (3.5%), for voriconazole, sensitivity is 84 (100%), SDD and resistance are nil, for caspofungin, sensitivity is 67 (79.7%), SDD 3 (3.5%), resistance 14 (16.6%). For amphotericin B sensitivity 78 (92.8%), SDD 2 (2.3%), resistant 4 (4.7%) (Table 3).

Antifungal susceptibility testing for *Candida* isolates were done by 'E' (Epsilon) test for the detection of MICs. Out of 84 isolates of *C. albicans,* 18 were resistant to fluconazole which were subjected to E test for MICs. Eighteen isolates are showing MICs were in the range of 6-7 µg/ml which are resistant. Total 6 isolates of *C. glabrata,* were resistant to fluconazole and were subjected to E test for MICs where the isolates were in the range of 48µg/ml which are resistant.

Out of 66 seropositive patients, viral load wherewith in the range of 1 lakh above, 16 seropositive patients where the viral load were in the range of ten thousand and ninety-nine thousand nine hundred and nine followed by 11 seropositive patients were within the range of thousand and nine thousand and nine hundred and nine, followed by 7 seropositive patients where the viral load were within the range of less than thousand (Table 4).

Table 3: Antifungal susceptibility for *Candida* isolates by disc diffusion method (n=100).

Species	N	Fluconazole			Itraconazole			Voriconazole			Caspofungin			Amphotericin B		
		S	SDD	R	S	SDD	R	S	SDD	R	S	SDD	R	S	SDD	R
C. albicans	84	69	15	3	54	4	16	84	-	-	67	3	14	78	2	4
	%	82.1	17.8	3.5	64.2	4.7	19.04	100			79.7	3.5	16.6	92.8	2.3	4.7
C. krusei	7	-	-	-	2	-	5	7	-	-	3	1	3	5	1	1
	%				28.5		71.4	100			42.8	14.2	42.8	71.4	14.2	14.2
C. glabarata	6	-	2	4	1	-	5	5	1	-	2	-	4	4	-	2
	%		33.3	66.7	16.6		83.3	83.3	16.6		33.3		66.60	66.6		33.3
C. tropicalis	3	3	-	-	2	-	1	3	-	-	2	1	-	3	-	-
	%	100			66.6		33.3	100			66.6	33.3		100		

Table 4: Distribution of viral load and CD4 count among the study population.

Viral load		CD4 count (cells/mm^3)		
Range	**Seropositive patients**	**0-200**	**201-500**	**>500**
100000 above	66	40	18	8
10000-99999	16	6	4	6
1000-9999	11	3	2	6
0-999	7	3	2	2
Total	100	52	26	22

Table 5: Distribution of *Candida* species in correlation with CD4 count.

Candida species	0-200	201-500	>500
C. albicans	43	21	20
C. krusei	4	2	1
C. glabrata	4	2	-
C. tropicalis	1	1	1
Total	52	26	22

C. albicans where CD4 count <200 cells/mm^3 are 40, followed by CD4 count 201-500 cells/mm^3 are 21, >500 cells/mm^3 CD4 count 20 in number. For *C. krusei* where CD4 count <200 cells/mm^3 are 4, followed by CD4 count 201-500 cells/mm^3 are 2 and >500 cells/mm^3 CD4 count isolates are 1 in number. For *C. glabrata* where CD 4 count is <200 cells/mm^3 are 4, CD 4 count 201-500 cells/mm^3 are 2 in number and CD 4 count >500 cells/mm^3 were no isolates of *C. glabrata.* Finally, for *C. tropicalis* single isolates for CD 4 count <200 cells/mm^3, 201-500 cells/mm^3 and >500 cells/mm^3 (Table 5).

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. Predominant isolate in the present study is the *C. albicans* 84 (84%) correlated with other studies like Franker et al where *C. albicans* 84 (84%) is the predominant isolate.[11] There is increasing incidence of non *albican Candida* species in oral Candidiasis and increasing rates of antifungal drug resistance particularly with the immunocompromised patients are seen in the present study, which are correlated with other studies like Hemachandran et al and Shymala et al shows an increasing incidence of non-albicans *Candida*. Hencerapid identification of candidiasis is important for the clinical management of immune-compromised patients.[12,13] In the present study, male preponderance was seen which are correlated with studies done by Anwar et al and Maheswari et al who studied the opportunistic infections spectrum and *Candida* profile in PLWHIV. Majority of the isolates in the present study were in the age group 31-49 years.[14,15] Studies have also reported the disease common in the age 25-49 years as HIV infection is most commonly seen in the sexually active age group. Sex preponderance in the present study are males 66 (66%) and females 34 (34%) correlated with Patella et al.[16] Among the non-albicans *Candida* spp., *C. krusei* was the most common species, a finding similar to Jayacharan et al [17] studies. 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.[18] 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.[19] In the present study, 82.1% of the isolates were susceptible to fluconazole. This result is similar to that reported by Swetha et al and Talukdar et al studies.[19,20] Itraconazole is used as an alternative to fluconazole for treating oral candidiasis. In this study the isolates of *C. albicans, C. krusei, C. glabrata* were 66%, 28%, 16% susceptible, and were resistant to itraconazole., 19%, 71%, 83%, 33% of *C. albicans, C. krusei, C. glabrata and C. tropicalis* 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. 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 led to the increase of reports of resistance.[21] Several authors have reported that apart from prolonged exposure, advanced immunosuppression is a major risk factor for azole resistance.[22] In the present study, among the 100 HIV patients with OPC, 70.7% had CD4 count statistically significant by proportional test ($p \leq 0.001$) This correlated well with the study conducted by Usha et al where 76.66% patients with OPC had CD4 count <200 cells/µl.[22] 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.

Limitations

Variation in patient backgrounds and patient care is the major limitation of the present study, as it may influence the outcome of the results. However, a large sample size can help to mitigate this limitation and provide more comprehensive findings.

CONCLUSION

C. albicans is the predominant isolate in the present study. *C. krusei, C. glabrata, C. tropicalis* are non albican *Candida* species were isolated. There is increasing trend towards non albican *Candida* species, hence speciation is necessary. Low CD 4 count and high viral load suggests significantly correlate with OPC in immunosuppressed patients especially in HIV. 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. Increasing drug resistance among non-albican *Candida species* indicating that there is need for speciation and antifungal susceptibility methods.

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