Spectrum of invasive candidiasis in correlation with CD4+ T lymphocyte count along with antifungal susceptibility pattern of isolates from PLHA patients recruited at a tertiary care hospital in Odisha

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Abstract

Introduction: Invasive candidiasis is considered as one of the major opportunistic fungal infections in PLHA patients causing significant morbidity and mortality in these patients with low CD4 cell count. This study was conducted to isolate and identify the various Candida species involved in invasive infections with their antifungal susceptibility pattern and to establish a correlation of opportunistic clinical presentations with patient immune status defined by CD4 cell count.

Materials and Methods: This prospective study included 231 HIV positive adult (>18yrs) patients with clinically suspected invasive fungal infections with CD4 cell count <500 cells/µl attending the (Antiretroviral therapy) ART centre. Samples were collected aseptically according to clinical presentations and were subjected to standard methods of identification to isolate and identify various Candida species. Further, antifungal susceptibility pattern was determined by Kirby Bauer disc diffusion method.

Results: Majority of patients in the study group were males in age group of 26-30yrs. Most of the invasive fungal infections in HIV positive patients were established to be Candidiasis among which oropharyngeal candidiasis followed by pneumonia were the most common clinical presentations. Most of the patients suffering from candidiasis belong to CD4 cell count ranging between 100-200cells/µl. *Candida* albicans followed by *Candida tropicalis* were the common isolates which were highly sensitive to Voriconazole and most resistant to Fluconazole.

Conclusion: A detailed understanding of epidemiology, immune status and antifungal sensitivities among HIV positive patients suffering from invasive candidiasis can alert the clinicians and help in timely diagnosis with appropriate treatment.

Keywords: PLHA (People living with HIV/AIDS), Opportunistic infections, Invasive candidiasis, CD4 cell count, Candida species, Antifungal susceptibility pattern.

Introduction

HIV/AIDS is a global pandemic causing significant morbidity & mortality. Globally, 36.9 million [31.1-43.9 million] people were living with HIV at the end of 2017.[1] India is reported to have the 3rd largest population with 2.1 million people suffering from HIV infection in 2018 with an incidence prevalence ratio of 0.04.[2] Approximately, 80% of these patients are seen to die mainly due to various opportunistic infections (OIs) rather than HIV which is the causative agent of AIDS.

Host immunity as well as environmental exposure play an important role in invasive fungal infections which are one of the commonest opportunistic infection in AIDS.[3] CD4+ T lymphocyte count and quantitative HIV RNA levels are most commonly used surrogate markers to determine the immune status of the HIV patients and categorize them on basis of associated clinical conditions.[4] CD4 helper T lymphocytes that direct and coordinate acquired immunity is greatly reduced in number and functionality thus predisposing the individual to various opportunistic infections.[5] The clinical course of HIV disease and pattern of OIs vary from patient to patient depending on immune status and different geographic locations. Patients with same CD4 count progress to AIDS at different times with various opportunistic infections occurring at different frequencies.[6]

Global recognition of the current medical importance of these human pathogens has increased the demand for information regarding their pathogenicity and the diseases they cause. So, they present as a wide range of infections starting from asymptomatic mucosal candidiasis to disseminated candidiasis presenting with pneumonia and meningitis.[5] Candida is a yeast like fungus causing candidiasis which is considered as one of the most common opportunistic infections in HIV/AIDS. *Candida albicans* is the most common isolate but with the emergence of other pathogenic non-albicans *Candida* (NAC) species, is Changing the clinical scenario along with antifungal susceptibility pattern is changing, thus making it necessary to isolate and identify the causative species.[7]

Available in 1990, Fluconazole is a well known fungistatic drug widely used in both prophylaxis and treatment of opportunistic infections ranging from mucosal candidiasis to invasive candidiasis. Emergence of resistant strains has led to the discovery of newer azoles and other group of antifungal drugs.[33]

Materials and Methods

The present study was carried out prospectively in the Department of Microbiology, MKCG Medical College & Hospital, Brahmapur, over a period of 24 months with the active assistance of the other clinical departments and ART centre.

Study group: Patients admitted to the in-patient department (IPD) or attending the ART centre are included in the study group. The study group comprised of 231 HIV

positive cases having clinically suspected fungal infections. Cases were selected basing on the inclusion criteria of -

1. >18yrs of age
2. Clinically suspected cases of fungal infection
3. Patients suffering from HIV infection / AIDS with CD4 cell count <500cells/µl

Patients Work Up

The detailed history of patient's illness, predisposing factors, history of exposure, other routine investigation reports pertaining to the disease were collected and entered into the performa. Determination of HIV status was confirmed by three tests (ELISA, i.e enzyme linked immunosorbent assay, Combaids-RS, HIV Tridot) in the Integrated Counselling and Testing Centre (ICTC) as per NACO guidelines. A total of 231 HIV positive patients were enrolled for the study after they provided informed written consent.

CD_4 Cell Count

CD_4 T cell counting was done using BD FACS Calibur™ System (Fluorescence Activated Cell Sorter). For each patient sample, one BD Trucount tube was labelled with the sample identification number. The BD Trucount bead pellet was verified to be intact and within the metal retainer at the bottom of the tube.20µL of BD Tritest CD3/CD4/CD45 reagent was added into the bottom of the tube, it was put just above the stainless steel retainer without touching the pellet. Using the reverse pipetting technique, 50µL of well mixed anti coagulated whole blood was put into the bottom of the tube. Care was taken, not to smear the sides of the tube with blood. The tube was capped and vortexed gently to mix. It was incubated for 15 minutes in the dark at room temperature. Meanwhile BC FACS Lysing solution, 10x was diluted 1:10(1x) with distilled water.450µL of this Lysing Solution was added to the tube and vortexed. It was incubated for 15minutes in the dark at room temperature. This sample was analysed on the flow cytometer.

Sample collection & transport: Most of the samples such as blood, orpharyngeal swab, oesophageal brushings, sputum, bronchial aspirate, bronchoalveolar lavage fluid, urine, stool, skin and nail were collected in duplicate in sterile universal container. All the samples were processed aseptically followed by isolation and identification according to standard laboratory operating procedures. Samples were collected in duplicate but both samples from single patient was counted as one sample.

All samples were subjected to microscopy by both Gram stain and KOH mount and were examined for budding yeast cells with pseudo hyphae (Fig. 1). All samples except blood, skin and nail were inoculated on duplicate sets of Sabouraud's Dextrose Agar (SDA) slants with Gentamicin.(Fig. 3) One kept at 37^0C in incubator & another placed at 25^0C inside the BOD (Biological oxygen demand) incubator. The tubes were inspected daily for the first week and twice a week for subsequent period till 21 days for the appearance of visible fungal colony growth. Blood was mixed into two BHI-agar biphasic medium (Fig. 4) and subculture also done on Blood agar plate (Fig. 2). Samples were also inoculated into the chromogenic media (Hichrome Candida differential agar media). It is a selective and differential media useful for presumptive identification of various candida isolates by producing pigmented colonies (Fig. 5). Biphasic blood culture media were observed for 6 weeks before they were declared as negative. The tubes showing yeast like creamy white pasty colony growth only at 37^0C were selected for further identification. The shape, size, colour, elevation and surface of the yeast like colonies were observed. A smear was prepared for staining with Gram's Method.The presence of Gram positive, budding yeasts of various shape and size from culture were taken into account. Those patients providing duplicate samples were only considered for the study where single type of *Candida species* was isolated from both the cultures in pure growth.

A Germ tube test was performed for presumptive identification of *Candida albicans* (Fig. 7). The suspected strain of *Candida spp.* was grown on cornmeal agar with Tween 80 (Dalmau culture plate method) and incubated at 25^0c. The formation of large, refractive, thick walled, terminal chlamydospores after 2 to 3 days of incubation were seen under high power microscope (Fig. 6). Typical morphology and arrangement of blastospores gives a presumptive identification of *Candida spp*. Further, Candida isolates were inoculated on Tetrazolium reduction medium (TRM) for presumptive differentiation between the species by reduction of tetrazolium compound shown by pigment production (Fig. 8).

Sugar fermentation and assimilation test:- Useful in identification of *Candida* isolates upto species level Sugar assimilation test (Auxanographic technique) was done by Disc impregnation – Pour plate Auxanographic method of Wickerham. Manually prepared sugar discs impregnated onto the surface of the agar plate are used and presence of growth around the disc is considered positive for particular carbohydrate. (Fig. 9)

Antifungal suceptibility testing done by Disc diffusion method included commercially available Himedia Mueller-Hinton agar supplemented with 2% glucose and 0.5 µg/ml methylene blue dye medium. PH of the medium was adjusted between 7.2 and 7.4 at room temperature after gelling. The inoculums were standardized to 0.5 McFarland. Discs of Fluconazole (25mcg), Itraconazole (10mcg), Amphotericin B (100units), and Voriconazole (1 mcg) were applied using the aseptic technique. Plates were incubated at 37^0C for 24 hours. Some strains where insufficient growth had occurred after 24 hours may need to be read after 48 hours incubation. The zones showing complete inhibition were measured and recorded. (Fig. 10)

Results

This study included 231 HIV positive patients. The demographic characterstics here projected the male: female ratio to be 1.7:1 Majority (23.37%) of PLHA patients in the study group were in the age group of 26-35years with a male predominance of 64.06%. (Table I) Majority of the

patients recorded in the study group were migrant labourers (68.83%). Maximum number of females (12.12%) were housewives showing the acquisition of HIV infection passively from their husbands (Table 2). In our study, oral thrush 159(68.83%) was the most common clinical presentation. Majority of symptoms in PLHA patients were seen within a CD_4 count range of 100-200 cells/µl (Table 3). Opportunistic fungal pathogens were found in 212 patients out of which *Candida species* were isolated from 133(57.57%) HIV positive patients. The species identified by standard methods included 82(35.49%) *C.albicans*, 27(11.68%) *C.tropicalis*, 12(5.19%) *C.glabrata*, 6(2.59%) *C.parapsilosis*, 4(1.73%) *C.krusei* and 2(0.86%) *C.kefyr*. Most (106/133) of the culture positive cases presented with oral thrush. (Table 4). Here, maximum fungal isolation were from HIV positive patients with CD4 count within a range of 101-200cells/µl (Table 5). The present study showed high 58(43.6%) fluconazole-resistance among the species. Most of *C.glabrata* isolates recovered in our study were innately less susceptible to fluconazole with resistant rates of 9/133(6.76%). The present study projected Voriconazole 128/133(96.24%) to be the most effective antifungal to be used against invasive *Candida* infections followed by Amphotericin B 119/133(89.47%) and Itraconazole 95/133(71.4%) (Table 6).

Table 1: Age & sex distribution in the study group (n=231)

AGE GROUPS (in yrs)	HIV/AIDS		Total
	Male	Female	
18-25	24(10.38%)	29(12.55%)	53 (22.94%)
26-35	54(23.37%)	32(13.85%)	86 (37.22%)
36-45	42(18.18%)	15(6.49%)	57 (24.67%)
46-55	19(8.22%)	5(2.16%)	24 (10.38%)
>55	9(3.89%)	2(0.86%)	11 (4.76%)
Total	148(64.06%)	83 (35.93%)	231 (100%)

Out of 231 HIV seropositive patients, 148(64.06%) males were more than females 83(35.93).The male to female ratio of total study group was found to be 1.7**:**1. Highest 86(37.22%) incidence was found to be in the age group of 26-35 yrs.

Table 2: Occupation wise distribution of cases in the study group (n=231)

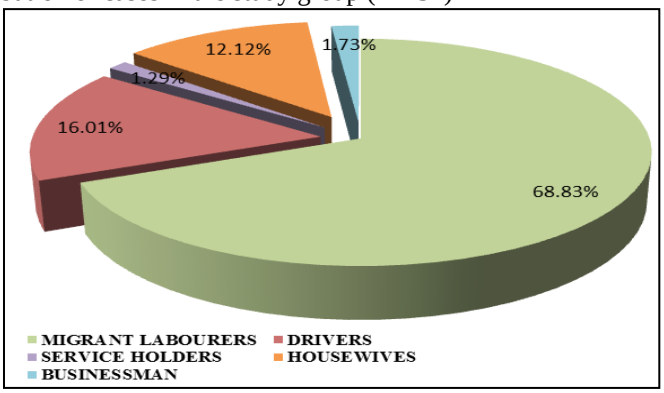


Table 3: Correlation of clinical presentation with CD4 count in HIV/AIDS patients (n=231)

Clinical Presentation	CD4 COUNT IN HIV/AIDS PATIENTS (n=231) (cells/µl)					Total Samples
	<100	100-200	200-300	300-400	400-500	
Oral thrush	18	54	46	26	15	159
Esophagitis	3	4	3	2	-	12
Diarrhea	2	14	8	13	5	42
Pneumonia	16	17	14	8	7	62
Meningitis	12	15	11	6	2	46
Septicemia	4	8	4	3	2	21
Lymphadenopathy	4	7	7	5	2	25
UTI*	2	3	3	1	0	9
Vulvovaginitis	-	1	2	3	1	7
Onychomycosis	-	-	3	2	2	7
Total	61	123	101	69	36	390

Total 390 samples were collected from 231 HIV/AIDS patients according to their clinical presentation. Majority of patients presented with oral thrush 68.83%(159/231) followed by pneumonia 26.83%(62/231) Most of the patients had more than one clinical presentation. This table also projects the various opportunistic conditions in correlation with CD4 count showing majority 31.53%(123/390) of these infections associated with CD4 count in the range of 100-200cells/µl. *UTI—Urinary tract infection

Table 4: Association of various clinical presentation with culture positive patients (n=133)

Clinical Presentation	Culture Positive Patients
Oral thrush	97
Oral thrush + Esophagitis	5
Diarrhea	8
Esophagitis + Diarrhea	2
Septicemia	1
Pneumonia	6
Pneumonia + Septicemia	1
Oral thrush + Pneumonia	4
UTI	2
Septicemia + UTI	-
Vulvovaginitis	2
Onychomycosis	5
Total	133

Most common presentation was oral candidiasis/thrush 106(97+5+4) followed by pneumonia 11(6+1+4) and diarrhea 10(8+2). Gastrointestinal system 116(97+5+8+2+4) was most common site from where Candida spp. were isolated in PLHA patients.

Table 5: Correlation of *Candida* isolates with CD_4 count in cells/µl in HIV/AIDS cases (n=133)

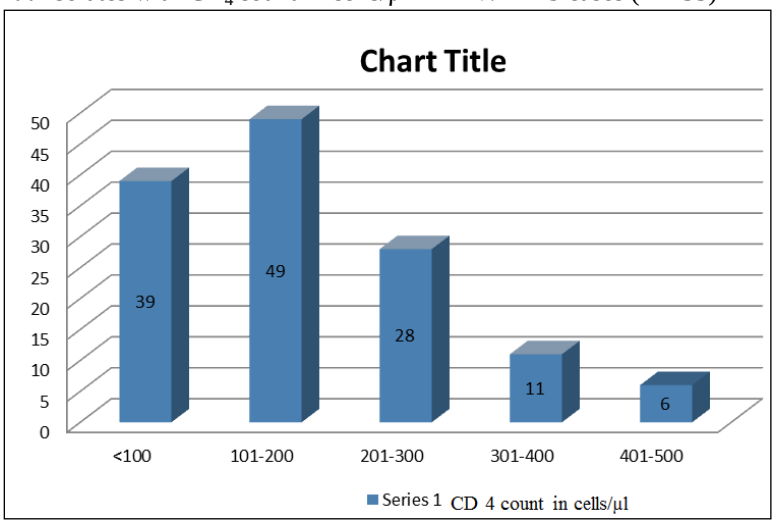


Table 6: Antifungal susceptibility pattern determined by Disc diffusion method (n=133)

Antifungals	S/R*	*Candida spp.*(n=133)	Percentage
Amphotericin B	S	113	84.96%
	R	20	15.03%
Fluconazole	S	81	60.9%
	R	52	39.09%
Itraconazole	S	87	65.41%
	R	46	34.58%
Voriconazole	S	128	96.24%
	R	5	3.75%

*S/R→S-Sensitive/R-Resistant

Among the antifungal drugs tested on 133 Candida isolates, maximum antifungal activity was shown by voriconazole (96.24%) followed by amphotericin B(84.96%). Majority (39.09%) of isolates were found to be showing resistant pattern against Fluconazole.

Figures

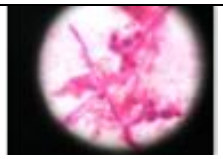


Fig. 1


Fig. 2

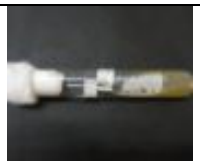


Fig. 3


Fig. 4


Fig. 5a

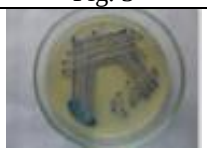


Fig. 5b


Fig. 5c


Fig. 5d


Fig. 6a

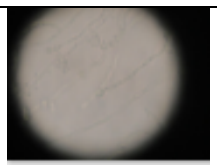
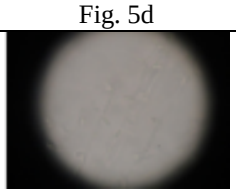


Fig. 6b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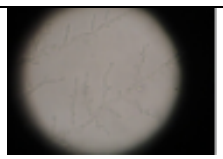
Fig. 6c

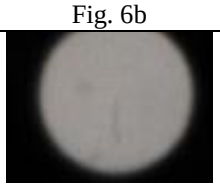
Fig. 6d

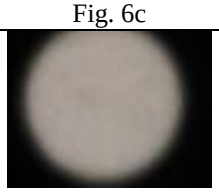
Fig. 6e

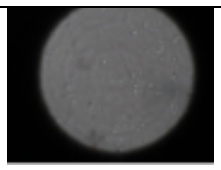
Fig. 7


Fig. 8

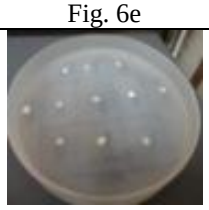
Fig. 9

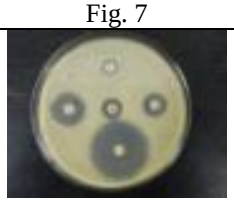
Fig. 10

Discussion

The present study was conducted in the Department of Microbiology in collaboration with other clinical departments and ART centre. The study was carried out over a period of 2 years with an aim to identify invasive candidiasis as opportunistic infections in 231 HIV/AIDS patients presenting with clinically suspected fungal infections. Their correlation with CD_4 cell count and antifungal susceptibility pattern was also determined.

The patients who acquire such infections have serious underlying medical conditions with most of the morbidity and mortality seen in immunocompromised patients. These opportunistic infections take advantage of the lowered cellular and humoral defences of the host. The ability to invade tissue will depend upon the overall immune status of the host and the suitability of the specific microenvironment for fungal growth. This is probably due to multiple factors which include the expanding group of persons with HIV infection that contributes to the increase in patients at risk for opportunistic infections. According to the Health & Family Welfare Department sources of Government of Odisha, Ganjam district tops the list of both HIV positive cases and AIDS related death cases. Most of the HIV cases from Ganjam district were reported from Polosara blocks.[8] Keeping in view the existence of diverse pattern of occurance, the present study was conducted to explore the pattern and trends of opportunistic infections by *Candida species* in HIV/AIDS cases.

The present study included 231 HIV/AIDS patients with male: female ratio of 1.7:1. This is in consonance with the national level statistics published by National AIDS Control Organization (NACO) in 2017 which highlights the data from 2015 depicting male:female ratio to be 1.5:1.[10] Slightly less (64.06%) male incidence compared to the scenario in the study done by Bhagyawatidevi et al. (94%) was seen.[9] Majority (23.37%) of PLHA patients in our study were in the age group of 26-35years similar to the data shown by Chandwani et al[14] but is in contrast to the study done by Patel et al.[11] where majority were in the age group of 20-29years (61%). This conflict may be due to the late reporting of associated opportunistic infections among these population.

Majority of the patients recorded in the study group were migrant labourers (68.83%) from Surat, Mumbai similar to study done by Chandwani et al.[14] Most of the infected females (12.12%) were housewives assuming that the acquisition of HIV infection was passively from their husbands proving that the most common method of HIV transmission was through heterosexual contact. This is in agreement with other studies reported by NACO (87.1%) and by Vajpayee *et al.* (59.8%) showing heterosexual transmission as commonest mode of infection.[12,13]

In our study, oral thrush 159(68.83%) was the most common clinical presentation similar to the reports by NACO and T.K Giri et al. but in contrast Kaur et al. have reported oral thrush as the second most common infection in AIDS patients.[14-16] Majority of symptoms in PLHA patients were seen within a CD_4 cell count range of 100-200 cells/µl. This is in accordance with the study done by both Delgado et al. and Ochiabuto et al. reporting higher prevalence of oral thrush in PLHA patients with CD_4 counts <200cells/µl.[17,18]

In the present investigation, all total 390 samples were collected from 231 HIV/AIDS patients and processed under proper aseptic conditions which accounted 159(40.76%) oropharyngeal swabs. Opportunistic fungal pathogens were found in 212 patients out of which *Candida species* were isolated from 133(57.57%) HIV positive patients which is very similar to the findings shown by Wadhwa et al. in his study who got 50% incidence of candidiasis in immunocompromised patients.[34] Similarly, Pruthvi et al. and Nalingeswaran et al. also reported prevalence of oral candidiasis to be 71% and 70% respectively.[19,20] Majority of *Candida species* were isolated from oropharyngeal swab cultures in duplicate. Majority of the studies from around the globe,[21,22] has shown a trend of shift in the species distribution of *Candida* in several major Indian hospitals. However, in our study *C.albicans* (35.49%) was found to be the most common isolate followed by *C.tropicalis* (11.68%) to be the second common isolated species and *C.glabrata* (5.19%) to be the third common species. Similar to our findings, Mokaddas et al. also found *C.albicans*(39.5%) to be the most common isolate.[23] *C.tropicalis* was the most frequently recovered non-albicans *Candida* (NAC) isolate in our study, while *C.glabrata* is the commonest non-albicans species worldwide.[24] Some of the studies done outside India like by Tercas et al.[25] and Viudes et al.[26] agree with our findings that among NAC, the most prevalent species were *C.tropicalis, C.krusei* and *C.glabrata*. The finding is also similar to Indian data provided by Singh et al reporting *C.tropicalis* to be an emergent pathogenic *Candida species*.[27] Our study has provided the correlation between isolation of Candida species and CD4 cell count of patient. Here, maximum isolation were from HIV positive patients with CD4 cell count within a range of 101-200cells/µl.

Pre exposure to antifungals, such as prophylaxis, in particular with azoles, has been associated with the occurrence of breakthrough infections with resistant *Candida* species. The outstanding feature in the present study was the alarmingly high 58(43.6%) prevalence of fluconazole-resistance among the species which is very similar to Leroy et al. study that reported a decreased (17%) susceptibility to fluconazole in the isolates.[28] Also, higher resistance in *C.albicans* has been attributed to the gradual emergence of non-*albicans Candida* species as a cause of refractory candidiasis, particularly in patients with advanced disease. Whereas *C. glabrata* and *C. krusei* have been classically observed in these settings, other resistant non-*albicans Candida* species are also being increasingly observed worldwide.[29]

C.glabrata has emerged as an important and potentially resistant opportunistic fungal pathogen. Most of *C.glabrata* isolates are less susceptible to fluconazole with resistant rates of 9/133(6.76%) similar to SENTRY survey done according to CLSI guidelines which also observed fluconazole resistance among *C.glabrata* isolates to be 5.6%. This SENTRY survey also found fluconazole resistance rate of 3.2% in *C.tropicalis* which is slightly lower than our study reporting resistant rate of 6/133(4.51%) due to increased emergence of this non *albicans Candida* in our setting.[30]

Antifungal drugs such as Voriconazole 128/133(96.24%) was found to be the most effective in use against invasive *Candida* infections followed by Amphotericin B 119/133(89.47%) and Itraconazole 95/133(71.4%). Some studies agree with the view that Voriconazole is an effective drug in those patients where isolated *Candida spp.* are resistant to both Fluconazole and Amphotericin B.[31,32] Amphotericin B susceptibility in our study is very similar to the susceptibility pattern described by Alves et al.[35]

Routine testing for antifungal susceptibility of clinical isolates is necessary to obtain baseline data & to observe any shift of sensitivity pattern in the population. This knowledge will surely help to monitor these patients effectively and create a treatment protocol for these opportunistic candidiasis in future.

Conclusion

The increasing number of immunocompromised patients has led to a rising burden of opportunistic fungal infections. Proper awareness and counselling will help the HIV positive patients to report at the very beginning of slightest symptoms. Complete follow up and time to time evaluation

of immune status by CD4 cell count should be promoted. Moreover, the widespread use of antifungal prophylaxis is changing the isolation pattern of fungi recovered in favour of resistant strains. This alarming rate of resistance should limit the use of this drug in high risk cases. The search for efficient drugs with fast action and less resistance should be the priority.

This observational study, aimed at characterizing the profile of invasive candidiasis in the setting of a typical southern Odisha hospital could further assist in alerting clinicians about the prevalence of this condition which can further promote the adoption of important prophylactic and treatment guidelines. Early suspicion with reporting and accurate treatment can bring down the incidence of opportunistic candidiasis in HIV/AIDS patients.

Conflict of Interest: None.

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