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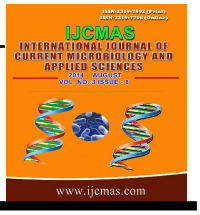

Original Research Article

Prevalence of Candida from Sputum in HIV infected Patients of Gujarat, India

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A B S T R A C T

The results of present study strongly supports the findings of other studies suggesting that since last two decades, *Candida albicans* had been replaced by Non candida albicans candida (NCAC) as primary and most important fungal pathogen in causing opportunistic fungal infections in HIV sero-positive patients. Candida were isolated and identified from sputa of 961 HIV sero-positive positive patients admitted at ART centre, and 300 HIV sero-negative patients as control group. The sputum samples from the both HIV positive and negative individuals were collected in a sterile container and processed by standard methods. Two consecutive samples at the interval of three days were collected. If two samples yielded the same growth, then only they were considered as positive for fungal pathogens. Out of 160(16.65%) Candida spp. isolated from HIV sero-positive patients, only Candida were isolated from 110(11.44%) samples, mixed infection of candia with bacteria in 50(5.20%) samples and no candida was found in 801 samples. *Non Candida albicans Candida* (NCAC) species predominate and detected in 97(10.1%)(63 pure candida + 43 mixed infections with Bacteria) isolates followed by *Candida albicans* species from 63(6.565%)(47 pure isolates+16 mix) samples. While in control group with HIV sero-negative patients out of total 19 isolates of candida spp only 2 were found to be NCAC with probability <0.001 when compared with isolates from HIV sero-positive patients. In the present study two (1.81%) cases of refractory candidiadis had been detected which is difficult to treat and may become increasingly unresponsive to antifungal therapy over time mostly caused in HIV patients

K e y w o r d s

Candida, Prevalence, HIV, Antifungal therapy, CD4 counts, HAART.

Introduction

The most common opportunistic fungal infection in HIV positive patients is candidiasis, affecting the mucocutaneous system mainly but the invasive form is also common. Resistance to azoles and other antifungal agents in the Candida species is a point of concern (Khan P Anawar et al, 2012). Oropharyngeal candidiasis(OPC), often the first sign of HIV infection, is the most prevalent fungal opportunistic infection in HIV-infected individuals.(Barr CE 1992) The spectrum of Candida

infection is diverse, starting from asymptomatic Colonization to Oropharyngeal Candidiasis (OPC), esophagitis,onychomycosis, vulovaginitis, cutaneous Candidiasis and systemic candidiasis or invasive candidiasis including candedemia. (Armstrong D 1989) A predominant source of morbidity and mortality among HIV positive individuals in late stages of HIV infection and low CD4 count below 500 dells/cumm, is opportunistic infection caused by agents that rarely infect immuno competent individuals (Jawetz et al., 2007). The occurrence of opportunistic fungal infections has risen progressively in recent years. Invasive fungal infections has been reported in recent years in 26% of chronically and intensively immunosuppressed patients (Topley and Wilson's, 2005). Infections with *Candida albicans* appear when CD4 count is between 500-200/cumm and may be the first indication of immunodeficiency.

The phagocytic cells and lymphocytes (T&B both) are believed to function together in protecting the host against fungal pathogens but the exact degree to which each is involved is not yet fully known. It has been shown that vegetative hyphal structures of *Aspergillus* and *candida* are ingested and killed by neutrophils (Jagdish, 2009).

Skin and mucosal surfaces play an important role in primary defence against pathogens. The muco ciliary action of mucus membrane is the prime clearance mechanism active against inhaled fungal spores. As the HIV positive individuals are prone to get recurrent respiratory infections, the mucosal barrier may be damaged and they are more vulnerable to develop fungal respiratory infections (Topley and Wilson's, 2005). The fungal infections depend on, exposure to sufficient inoculum size of organism and general resistance of the host

With introduction of antifungal agents, the cause of candida infections shifted from *C. albicans to C. glabrata* and other non albicans species, *as C. glabrata* and *C. krusei* develop resistance to fluconazole (Topley and Wilson's, 2005). Fungal infections may disseminate and cause fungaemia, which is a grave condition in immunosuppressed individuals. Thus, prompt diagnosis by standard microbiological methods and treatment are crucial.

Lower respiratory tract infections [LRTI] include infection of the trachea, bronchi, lungs eg tuberculosis, whooping cough, pneumonia etc. Of all the known pathogens of LRTI, tuberculosis is considered to be the most fatal infection with elaborate complications. Tuberculosis is an ancient human disease that has long been a major public health challenge in the world, predominantly in the developing countries, with one third of the world population affected across & 8 million new cases every year (Latha.R,et al, 2011). Tuberculosis has always seemed to be associated with many other secondary infections, the commonest among them being *Candida spp* infection. *Candida* species are often found in sputum specimens suspected of tuberculosis. Their role as a possible cause of pulmonary disease is a frequent consideration, particularly in patients receiving immunosuppressive or long-standing antimicrobial therapy (Masur H, et al,1977). Though *Candida* is not a significant clinical factor in pulmonary diseases, it causes serious complication as coinfection with other chronic illness(Phukan AC, 2000).

Candida albicans was considered as the most important pathogen among the *Candida spp* until emergence of HIV, now the steady increase in the recovery rates of other species like *Candida krusie,* Candida

tropicalis, Candida glabrata, and *Candida paropsilopsis*. The reason for the increase may be linked to generalized increase in mycoses, increase in the invasive medical procedures, use of wide spectrum antibiotics, use of immunosuppressive drugs and last but not least HIV. Hence the longevity of hospitalization of the severely immunocompromised individual(HIV patients) has allowed these species to emerge and cause diseases (Liu ZY, Sheng RY et al,, 2003). Identification of *Candida* to the species level has become mandatory to aid the selection of appropriate antifungal agents in treatment of invasive candidiasis because most of the Non Candida albicans Candida [NCAC] usually exhibit a reduced susceptibility to the common antifungal agents especially *Candida glabrata* which exhibits reduced fluconazole susceptibility(Liu ZY, Sheng RY et al,, 2003, Elena Eraso, et al,2006).

The low absolute CD4+ T-lymphocyte count has traditionally been cited as the greatest risk factor for the development of OPC and current guidelines suggest increased risk once CD4+ Tlymphocyte counts fall below 200 cells/μL. OPC remains more common in HIV infected patients than those with a similar degree of immunosuppression (bone marrow transplant or patients receiving chemotherapy).

This observation suggests that HIV itself may play a role in host susceptibility (Calderone RA. 2001). The defect in cellular immunity characteristic of HIV infection predisposes to Candida infections. Role of lymphocytes and polymorphonuclear leukocytes in defense against infection with Candida is of great importance, as indicated by the relatively common occurrence of disseminated candidiasis in neutropenic patients without defects in cellular immunity (Armstrong D.1989).

The present study had been conducted primarily to compare the prevalence of Candida albicans and NCAC in fungal opportunistic infections seen in HIV sero-positive patients and secondarily to compare their prevalence even with HIV sero-negative patients with the infections with candida species. It also elaborate the importance of HAART in maintaining high level of CD4 counts which it self had reduced or controlled these opportunistic infections with candida species.

Materials and Methods

This study has been conducted at the ICTC & ART Centre, New Civil Hospital and Government Medical College, Surat. All the cases and both control group patients (961 cases and 300 group of control patients) included in the study were those who were either OPD or indoor patients of New Civil Hospital, Surat, with complaint of respiratory tract infections(Cough persistent for more than a week).

Case Definition for T

Cases was defined as, patients with both HIV sero-positive as well as suffering from respiratory tract infections (RTI) at the time of data collection. One patient was included only once.

Definition for C1(Control Group)

C1 Control group was defined as patients with respiratory tract infection (RTI) but sero- negative for HIV at the time of data collection.

Processing of Samples

Two consecutive sputum samples at an interval of 3 days were collected from HIV the patients and their CD4 counts were

presented in Table 3. All patients complained of cough and fever for more than one week.

Sputa samples were collected in a sterile wide mouthed container. Patients were asked to wash their oral cavity with distilled water before collecting sputum in order to avoid contamination of sputum with commensal flora from oral cavity (Koneman color Atlas and Text book of Diagnostic Microbiology, 1997). Specimens were processed by doing Gram's staining for direct smears and KOH mount. Gram's stained smears were examined under oil immersionobjective of microscope for the presence of inflammatory cells (pus) cells and fungal elements.

Quality of the sputum was assessed by examining Grams staining smears. The specimen was considered as acceptable when number of squamous epithelial cells are less than 10/ LPF (Bailey and Scott's, 2002; Koneman color Atlas and Text book of Diagnostic Microbiology, 1997). The samples that showed fungal elements and pus cells in direct Gram's staining were processed further (Koneman color Atlas and Text book of Diagnostic Microbiology, 1997; Topley and Wilson's, 2005).

Sputum was inoculated on two sets of Sabouraud's dextrose agar (SDA with antibiotic gentamicin alone and SDA with gentamicin and cycloheximide) and incubated at 25°C in BOD for 4 weeks. SDA bottles were examined for growth once in two days during 1st week and twice a week thereafter up to 4 weeks. SDA medium with growth was processed by standard methods (Mackie and McCartney, 2008).

Mucoid, yeast like growth was processed by doing Gram staining, capsular staining, germ tube test,. Gram's staining was done to all the isolates with mucoid and yeast like growth and observed for gram positive budding yeast cells.

Germ tube test: All candida isolates were tested for germ tube formation. A colony was inoculated in human serum and incubated at 37°C. After 2 to 4 h wet mount was prepared and observed for germ tubes.

Results and Discussion

In this study 110(11.45%) patients with HIV(T Group) were presented with pure monomicrobial candidal isolates, while HIV sero-negative group (C1 Group) showed only 16(5.33%) fungal isolates (Table 1). From this result it seems to be very clear that with decreasing CD4 below 500 cell/μL, even risk of getting fungal infections in HIV patients increases more than two fold. There are no detectable differences in the virulence of strains isolated from HIV-infected or HIV-uninfected persons (Korting HC et al,1988). Recurrent disease can result from the same or from different species or strains of Candida. (Schmid J et al,1992) The emergence of different strains or species is more likely in persons with lower CD4 lymphocyte counts and exposure to antifungal therapy(Powderly WG,et al,1992).

In the present study poly-microbial infections (Mixed infections) were seen in 50 (5.20%) patients with HIV, but they were seen only 3(1.0%) patients in HIV sero-negative group (Table 2).

A number of factors are important in the development of mucocutaneous candidiasis.(de Repentigny et al, 2004) The level of immunosuppression is paramount.(Steele C, Leigh J, et al, 2000) Other host factors important in the defense of Candida

infections include blood group secretor status (such as presence or absence of specific Lewis antigens), salivary flow rates, condition of the epithelial barrier, antimicrobial constituents of saliva, presence of normal bacterial flora, and local immunity.(McCarthy GM et al,1991; Diz Dios P, Ocampo A et al, 2001) Several studies suggest an impairment in a number of anti-Candida host defence mechanisms in persons with HIV infection. (McCarthy GM et al,1991; McCarthy GM, 1992; Yeh CK, Fox PC et al, 1988) Higher levels of HIV-1 RNA in the plasma also have been associated with increased rates of mucocutaneous candidiasis and colonization with Candida (Cauda R, Tacconelli E, et al, 1999; Gottfredsson M, et al,1999).

The fungal infections depends on exposure to sufficient inoculum size of organism and general resistance of the host. With introduction of antifungal agents, the cause of candida infections shifted from C. albicans to C.glabrata and other non-albicans species (NCAV), as C. glabrata and C. krusei develop resistance to fluconazole(Topley and Wilson's, 2005). Fungal infections may disseminate and cause fungaemia, which is a grave condition in immunosuppressed individuals. Thus prompt diagnosis by standard microbiological methods and treatment are crucial.

The mean age of all the 961 patients of group T (HIV +ve/TRI +ve) was found to be 33.94+ 09.54 years, while mean age of patients with positive fungal isolates was also 34.03 +12.86 years. Khan et al,(2012) even reported mean age 32 years in their study. The more number of the patients seen in the two age groups(21-40 years) was due to more number of the patients even seen in group T. It is suggestive that not any specific trend had been observed between age and fungal infections in HIV sero-positive patients. The mean age in C1 group patients was found to be 35.26 + 20.06 years.

In the present study Candida albicans were isolated from 4.89% (47/961) of HIV-infected patients with RTI of group T. This matches well with the findings of V.V. Shailaja et al,(2004), who also found 5 % C.albicans in their study, while they found 3.33% C.albicans from HIV-uninfected patients of RTI, in the present study also C. albicans were isolated from 1.33%(4/300) of HIV-uninfected patients.

Non-albicans Candida were isolated from 29% of patients while C.albicans were isolated from just 26% of patients in study of Bharathi M and Usha Rani(2011),which showed the change in the trend of candida infections towards non albicans spp. In the present study also nonalbicans Candida were isolated in 6.56%(63/961) HIV-infected patients, while C.albicans were isolated from 4.89%(47/961) patients of the same group proved changed trend of candida infection towards non-albicans Candida. This changed trend is due to resistance to fluconazole developed by non-albicans spp. like C.krusei and C.glabrata. As fluconazole is commonly used antimycotic drug for prophylaxis. Shailaja et al(2004) in Hyderabad (India) also isolated more number of non albicans spp. in 18 cases than C. albicans in 6 cases.

Aruna Aggrwal et al(2005), isolated C.albicans in 20(62.5%) out of 32 isolates in Punjab. Jha et al.(2006) from Khatmandu isolated 20 C.albicns and 10 non albicans from 462 samples of lower respiratory tract infections. Prasobh et al.(2009) performed resistotyping of 350 Candida albicans isolated from sputum samples in Tamilnadu. All the above studies were geographically

from the same region. C. albicans was isolated from sputum samples in all these studies. In the study of opportunistic fungal infection in AIDS patients by Rakhamanova et al(1998), the culture positivity for C. albicans and C.neoformans in pulmonary infections was 4% each. Yongabi et al (2009) isolated 12 strains of C.albicans from 98 sputum samples. Non albicans spp predominate in the study by Shailaja et al(2004) and Bharathi M et al(2009)and in the present study, where as C. albicans was found predominate species in Jha et al(2006) study. In some other studies only C. albicans was mentioned. But over all, the importance of C. albicans as causative agent in pulmonary infection was proved by all these studies.

In our study, candidial infection was found to be the most common (16.96 %) opportunistic fungal infection. Even Mulla et al (2007) from the same geographical region reported candidiasis in all most same 19.44 % from HIV positive patients. Other studies even reported candidiasis in 23 to 27% of HIV positive patients ((Sharma SK et al, 2003; Ismail H. Sahand et al,2009.). .But Pruthvi *et al*. (2006) and Khan (2012) reported very high prevalence of candidiasis in about 71 % of HIV positive patients, Nagalingeswaran K *et al*. (2000) in 70 %, Singh A. *et al*. (2003) in 65 % and AnupriyaWadhwa *et al*. (2003) found candidiasis in 50% of the HIV positive patients. The reason for their higher isolation rates might be due to firstly as all of them might had used throat swabs instead of sputum as used in the present study and secondly might be due to demographic and regional differences which also affect the spectrum of illness seen in different environment along with exposure to various predisposing factors.

Several mechanisms may contribute to in vitro resistance to antifungals. Some yeasts have single-drug resistance, whereas others are multidrug resistant. Azole resistance has been demonstrated in yeasts that contain alterations in the enzymes that were the target of azole action or were involved in ergosterol biosynthesis. The cytochrome P450-dependent 14alpha-sterol demethylase (P450DM) and the delta(Katz MH, Greenspan D,1992; Tavitian A, Raufman JP, et al, 1986) sterol desaturase are enzymes that, when altered, result in azole resistance.(Hitchcock CA.1993; Vanden Bossche H,1994) Reduced cell permeability is another mechanism of azole resistance.(Ryley JF, Wilson RG 1984) Finally, active efflux of drug also has been observed.(Parkinson T, et al,1995) The prevalence of these mechanisms, however, is unknown. Further, it is not clear whether certain mechanisms of resistance may be overcome by increasing the dosage of the drug.

Refractory fungal infection is defined as the failure to respond to antifungal treatment with appropriate doses for a standard duration of time (eg, 14 days). (Fichtenbaum CJ et al, 2000; Arilla MC et al,,1992) Fluconazole-refractory disease has received particular attention because of significant morbidity, treatment often requiring the use of parenteral agents, and the frequency with which fluconazole has been prescribed. The annual incidence of fluconazole-refractory OPC(Oropharyngeal candidiasis) was reported to be 4-5% in advanced HIV infection in developed countries prior to the introduction of combination ART. (Fichtenbaum CJ et al, 2000;Sha B. et al, 1993) Like most other opportunistic infections, fluconazole-refractory OPC is less common with the widespread use of effective ART. Candidiasis refractory to amphotericin B is exceedingly uncommon.(Fichtenbaum CJ, Powderly WG ,1998) Of note, clinical

failures also may result from inadequate drug absorption or drug interactions that decrease the levels of some antifungal medications. (Kaltenbach G,et al,1996)

In the present study only two(1.81%, 2 out of 110 pure candidal infections) cases of refractory candidiadis were noted. Refractory candidiasis often is difficult to treat and may become increasingly unresponsive to therapy over time. The most important step is to determine what medications and dosages have been tried and whether adherence to therapy has been adequate. Removing any interacting medications or increasing the dose of the antifungal agent may be curative in some persons. In general, persons with OPC that is unresponsive to clotrimazole, nystatin, ketoconazole, or itraconazole tablets will respond to fluconazole. Persons with OPC unresponsive to fluconazole 200 mg daily given for 2 weeks are less likely to respond to higher doses but sometimes do respond. Additionally, flucytosine may be added for synergy.

Among the LRTIs, tuberculosis has long been a major public health challenge in developing countries. Candida infection has been the most important secondary infection in HIV patients. *Candida albicans* was considered to be the only species of real medical importance and the other were considered as occasional pathogens. Over the past 2 decade many of the Non Candida albicans Candida [NCAC] have emerged as significant pathogens of the clinical importance especially in immune suppressed HIV patients. Candida species are diverse and this diversity range of Candida has provided new challenges in the diagnosis, treatment of Candida and also in the study of their virulence & biology(Liu ZY, Sheng RY,2003).

The first step in the development of a candida linfection is colonization of the mucocutaneous surfaces. HIV infection is not only associated with increased colonization rates but also with the development of overt disease. During the course of HIV infection, the rate of Candida infection is inversely related to the CD4 counts of the patient which in turn depends on the use of Anti-retroviral treatment(ART).

Out of 160(16.64%) patients with candidal infection in the present study, only 41 were on HAART (ART). The improvement in CD4+ cell counts of patients with candidial infection who were on ART was maximum in the regimen of the combination of Zidovudine, Lamivudine and Nevirapine followed by Zidovudine, Lamivudine and Efaviranz (Table 3).

The occurrence of opportunistic fungal infections has risen progressively in recent years. Invasive fungal infections had been reported in 26% of chronically and intensively immunosuppressed patients (Topley and Wilson's 2005).Infections with Candida albicans appear when CD4 count is between 500-200cells/µl and may be the first indication of immunodeficiency. In the present study the mean of CD4 count for C. albicans was found to be 257.12+ 82.86 cells/µl, while the mean CD4 count for non-albicans Candida was found to be 499.73+196.24 cells/µl in HIV-infected patients with RTI of group T, which had been even well supported by findings of Jawetz et al.(2007).

Changes in HIV therapy initiation guidelines affect clinicians, patients, and policymakers who continue to search for the most efficient and effective treatment strategies. Prior to 2013, initiation was recommended at 350 cells/µL (WHO,2010) and developing countries—where governments and international agencies play a greater role in

HIV management due to low per capita income on the part of patients are still adjusting to these changes. The WHO and USDHHS in 2013 recommended starting therapy at >500 cells/μL based on the scientific body of science for HIV clinical research. Some support for this change in recommendation may be provided through this meta-analysis of studies that compares the former recommendation (initiation at <350 cells/μL) to the new recommendation (>500 cells/μL) but only when <200 cells/μL are used as a referent group (Babatunde Olubajo et al.2014).

In resource limited countries, like ours where increasing the threshold for initiating treatment to CD4 counts of 350 cells/μL is financially very difficult, the WHO guidelines may be met with resistance and consequently may not be adopted and adhered to. The review of Babatunde Olubajo et al (2014) gives insight to the risk associated with maintaining a low CD4 threshold as compared to the elevated threshold recommended by WHO and the USDHHS. Results from their review indicated a greater risk in those initiating therapy at 350–500 cells/μL compared to those initiating therapy at CD4 >500 cells/μL, but only with the studies that were appropriate for combining the effect. Of the studies with <200 cells/μL as a referent group, there was a pooled 11% elevated risk for the 350–500 cells/μL cells; only one of the three studies exhibited a decreased risk for initiating therapy at 350–500 cells/μL as opposed to initiating at >500 cells/μL(Babatunde Olubajo et al.2014).

In the present study mean CD4 count of the all patients was found to be 339.10 + 84.0 cells//μl, but when 160 patients with candida species were excluded from 961 patients of group T, this group without candida consisted of 801 patients and their mean CD4 count was found to be 437.26 cells//μl,. If cumulative mean of CD4 count of both Candida albicans and NCAC was calculated together, it was found to be 404. 20 cells//μl. As recommended by WHO(2013) and many recent studies that HAART should be started very early around at CD4 count of 500 cells//μl, instead of standard HAART in which HAART is started around 200 or 350 cells//μl, the mean CD4 count of NCAC in this study is 499.73 + 84.0 cells//μl and above two mean CD4 altogether strongly support other studies which recommended HAART should be started at 500 cells//μl, according to this study to stop opportunistic candidal(NCAC) infections in HIV infected patients (Table 4).

HIV infection is no longer characterized by high morbidity, rapid progression to AIDS, and death as when the infection was first identified. While anti-retroviral drugs have improved the outcome of AIDS patients, clinical research on the appropriate time to initiate therapy continues to evolve. Optimal therapy initiation would maximize the benefits of these drugs, while minimizing side effects and drug resistance. Recent 2013 WHO guidelines changed HIV therapy initiation from 350<cells/μL to 500>cells/μL. Most individual study comparisons showed a benefit for starting treatment at 500 cells/μL in comparison with starting at the 350–500 cells/μL range with risks ranging from 19% to 300%, though a number of comparisons were not statistically significant. Overall, the study provides evidence based support for initiating anti retroviral therapy at cell counts >500 cells/μL wherever possible to prevent first fungal opportunistic AIDS related morbidity and subsequent mortality..

Table.1 Candidal profile of patients of both the groups studied (HIV reactive and HIV non-reactive patients)

Pathogenic Isolates	HIV +VE RTI +VE (T) patients(n=961)		HIV -VE RTI +VE (C1) patients (n=300)		P Value
	(n)	%	**(n)**	%	
Candia albicans	47	4.89	15	5.00	<0.05
Non Candida albicans Candida (NCAC)	63	6.56	01	0.33	<0.02
Pure Candidal Infection	**110**	11.45	**16**	5.33	<0.01
Total Candida infection Pure+Mix	**160**	16.65	**19**	6.33	<0.001

Table.2 Polymicrobial fungal isolates from patients of both the groups

Microorganisms	HIV +VE RTI +VE (T)		HIV -VE RTI +VE (C1)	
	(n)	%	**(n)**	%
M. tuberculosis + Candida (NCAC)	17	10.25	1	6.3
M. tuberculosis + Candida albicans	6	3.75	1	6.3
Klebsiella pneumoniae + Candida(NCAC)	9	5.63	0	-
Klebsiella pneumoniae + Candida albicans	5	3.13	0	-
Pseudomonas + Candida (NCAC)	4	2.5	0	6.3
Pseudomonas + Candida Albicans	3	1.88	1	-
Proteus + Candida (NCAC)	4	2.5	0	-
Proteus + Candida albicans	2	1.25	0	-
Total polymicrobial(Mix) infections	**50/961**	**5.20**	**3/300**	**1.00**

Table.3 Improvement of CD4+ count in different ART Regimen

Drugs included in the regimen	Baseline CD4 cell count (mean)	CD4 cell count at 6 months (mean)	CD4 cell count at 12 months (mean)
Zidovudine+Lamivudine+ Nevirapine	123	198	261
Stavudine + Lamivudine + Nevirapine	147	193	201
Zidovudine + Lamivudine + Efavirenz	137	189	250
Stavudine + Lamivudine + Efavirenz	119	181	223

Table.4 Comparison of Mean CD4 of patients of various groups

Organisms	Mean CD4	Standard Deviation	Isolates No.	%
Candida albicans	257.12	+82.96	63	6.55
Candida (NCAC)	499.73	+196.24	97	10.09
Candida albicans + Candida(NCAC)	404.20	+193.14	160	16.65
Other patients excluding only Candida+NCACs	437.26	+231.5	801	83.35
Total Patients	339.10	+ 84.0	-	-

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