ORIGINAL ARTICLE

A study of antifungal drug sensitivity of Candida isolated from human immunodeficiency virus infected patients in Chennai, South India

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ABSTRACT

Background: The purpose of this study was to study the drug sensitivity pattern of Candida seen in HIV seropositive patients in Chennai, South India. **Materials and Methods:** 36 oral rinse samples were collected from HIV seropositive individuals with (21 patients) and without (15 patients) clinical candidiasis. The type of Candidiasis, quantitative estimation, differentiation of candida species and antifungal susceptibility testing was done using different tests. **Results:** In the 21 patients with candidiasis, pseudomembranous type predominated with low CD4 counts and high colony forming units. Antifungal Drug sensitivity test revealed resistance to fluconazole which is attributed to long term exposure to the drug. **Conclusion:** The results of the study confirm the hypothesis that candidal species can be isolated in HIV positive patients with clinical candidiasis. In HIV infection there are fluconazole resistant candida species emerging mainly due to long term exposure to the drug.
Key words: HIV, Candida, colony forming units, CD4 count, antifungal drugs

INTRODUCTION

The incidence of oropharyngeal candidal infection has increased as a part of the Acquired Immunodeficiency Syndrome (AIDS) epidemic. This fungal infection may occur as the first sign of the Human Immunodeficiency Virus (HIV) infection or at times may be the patient's only chief complaint. Oral candidiasis reflects a declining immune system signaling the terminal phase of HIV infection. Increase in retroviral replication and an associated decline in immune defenses render these 'at risk' patients particularly susceptible.[1]

Candida organisms are normal inhabitants of the human gastrointestinal (GI) tract and may be recovered from up to one-third of the mouths of normal individuals and two-thirds of those with advanced HIV disease. Oral colonization with inherently drug-resistant organisms is more common in advanced HIV infection. Candidiasis is treated with antifungal medications, which include polyenes such as nystatin and amphotericin, imidazoles such as clotrimazole, and triazoles such as fluconazole and itraconazole. In HIV-infected patients, fluconazole, a water soluble bis-triazole, is advantageous over other antifungals because of its excellent tolerance level, low toxicity, and favorable pharmacokinetics.[2]

Fluconazole commands one-fourth of the drug market for antifungals in the world. With such high levels of exposure, reports of treatment failure, relapse, and resistance have begun to appear. The development of drug resistance is related to low CD4 lymphocyte count and prolonged exposure to the drug. An important mechanism in the development of resistance is replacement of the fluconazole-susceptible *Candida albican* strains, with other species like *C. glabrata* and *C. krusei,* which are intrinsically less fluconazole-sensitive.[3]

The rise in the incidence fungal infections has exacerbated the need for the next generation of antifungal agents, as many of the currently available drugs have undesirable side effects, are ineffective against new or re-emerging fungi, or lead to the rapid development of resistance.

The aim of this study was,

- to isolate and speciate candida in HIV-seropositive patients, with and without clinical candidiasis, in Chennai, South India
- to estimate the colony forming units (CFU's) in patients with candidal growth

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- to ascertain antifungal drug sensitivity of candida for the following drugs: Fluconazole, itraconazole, nystatin, amphotericin B, and clotrimazole

MATERIALS AND METHODS

Patient selection

A total of 36 patients with a complete case history were examined and grouped under two categories.

Group 1 — This study group consisted of 21 HIV-seropositive patients who had clinical candidiasis. Candidiasis was confirmed by smears.

Group 2 — This study group consisted of 15 HIV-seropositive patients who had no clinical candidiasis.

All these patients were confirmed HIV-seropositive by ELISA and Western Blot (ERBA, USA and QUALICOD, USA)

Sample collection

Oral Rinse Technique — The patient was asked to rinse 15 ml of physiological saline vigorously in the mouth for 30 seconds and the same was collected and labeled in a sterile uricol container. The uricol container was kept in an ice flask at 4˚C. The samples were taken to the microbiology laboratory immediately, for conducting the experiment.[4]

Laboratory methods

Quantitative estimation of Candidal colonies

One hundred microlitres of the mouth wash samples were inoculated on sterile Saboraud's Dextrose Agar (SDA) plates and surface streaking was done, for the estimation of the CFU count. The plates were incubated overnight at 37˚C and observed the next day. The number of colonies was counted and the average was calculated. The counts were expressed in CFU/ml. The presence of candida was confirmed by the presence of creamy white colonies. It was reconfirmed by gram staining and observing the presence of ovoid yeasts.

Differentiation of Candida species

The Candidal species were identified by biotyping. It is the identification of the candida species according to their biochemical characteristics. The various tests that were done under the conventional method of biotyping were:

Sugar fermentation test [Figure 1]

Sugar assimilation test [Figure 2]

Germ tube production test [Figure 3]

Chlamydospore production test [Figure 4]

Antifungal susceptibility testing by the disk diffusion method

The media used was Bacto yeast Nitrogen Base Part A with L-Asparagine and glucose sterilized by means of the membrane filtration method. Part B was prepared by using sodium dihydrogen phosphate, dipotassium hydrogen phosphate, and agarose melted by heating from 90˚C to 100˚C for 10 minutes, and the pH adjusted to 7.

The isolates were subcultured on Sabouraud Dextrose Agar (SDA) and incubated at 37˚C overnight. Saline suspension of the organism was made and the turbidity was adjusted to 0.5 Mc Farland standards. A lawn culture was done on freshly prepared Yeast nitrogen base agar plates.

The disks were placed on the media and the plates were incubated at 37˚C. The zone of inhibition was recorded after 24 hours and 48 hours. The isolates were classified as susceptible, dose-dependant, and susceptible and resistant, based on the diameter of the zone of inhibition [Figures 5 and 6].[5]

RESULTS

A total of 36 oral rinse specimens were collected from HIV-seropositive individuals with and without clinical candidiasis (21 patients had clinical candidiasis and 15 patients did not have clinical candidiasis). Table 1: The number of males in Group 1 was 19 with a mean age of 32.3 years (range 21 – 45) and two were females with a mean age of 34.5 years (range 19 – 50).

The number of males in Group 2 was 13 with a mean age of 34.2 years (range 27 – 47) and two were females with a mean age of 28 years (range 22 – 44). Table 2: Of the 21 patients with clinical candidiasis, pseudomembranous candidiasis was seen in 16 patients, and erythematous candidiasis in five patients. Table 3: Of the 16 patients with pseudomembranous candidiasis, CD4 counts were available for nine patients. Of the nine patients, four patients had CD4 $\leq$ 200 and five patients had CD4 > 200. Five patients had erythematous type of candidiasis. The CD4 cell count was available for all the five patients, one patient had CD4 $\leq$ 200 and four patients had CD4 > 200. Table 4: Group 1 Patients with clinical candidiasis, *Candida albicans* species was isolated in 19 (90%) patients and *Candida krusei* was isolated from two (10%) patients. Group 2 patients without clinical candidiasis showed no evidence of growth. Table 5: In patients with pseudomembranous candidiasis the colony forming unit/ml, ranged from 200 – 43200/ml (mean 9175/ml) and in the erythematous type the colony forming unit/ml ranged from 700 – 11000/ml (mean 5040/ml). Table 6: Mean CFU/ml of 11280 was seen in five patients in the CD4</ = 200 group. Mean value was 97.4 cells/mm^3. Mean CFU/ml of 3533 was seen in patients in the CD4> 200 group. Mean value was 560.2 cells/mm^3.

Table 7: Of the 21 patients with clinical candidiasis, *Candida albicans* was isolated in 19 patients and in two patients *Candida krusei* was isolated. In the 19 patients with *Candida alblicans*, fourteen (73%) strains were sensitive, one (6%) was dose-dependent susceptible, and four (21%) were resistant to fluconazole. All the 19 (100%) were sensitive to nystatin,

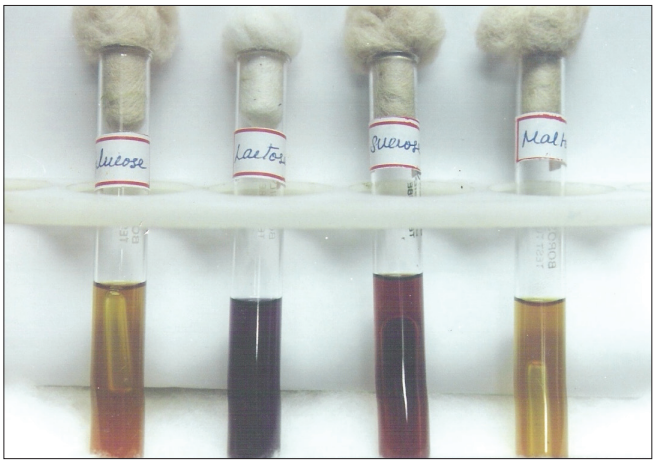

Figure 1: Sugar fermentation test showing reaction for *Candida albicans*

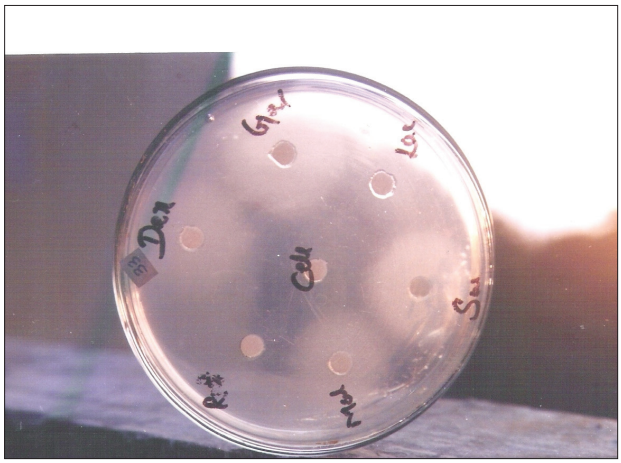

Figure 2: Sugar assimilation test showing reaction for *Candida albicans*

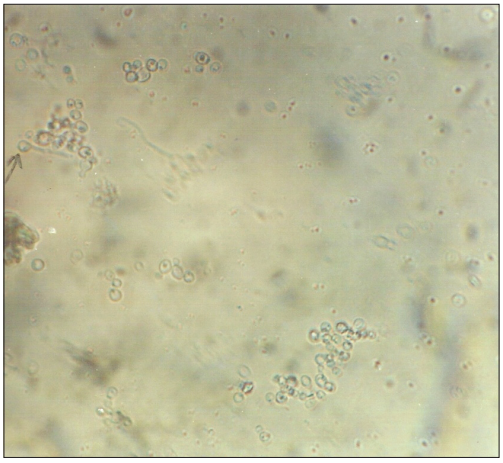

Figure 3: Positive germ tube test

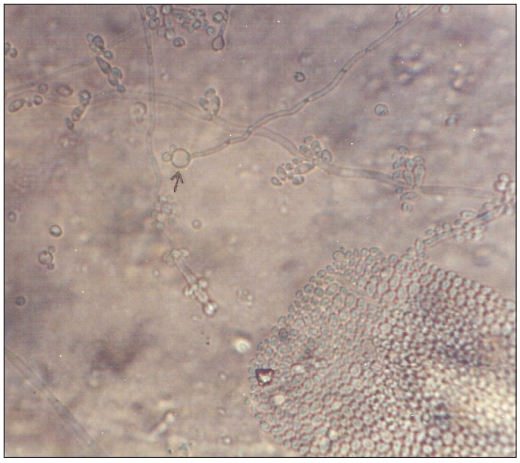

Figure 4: Positive chlamydospore test

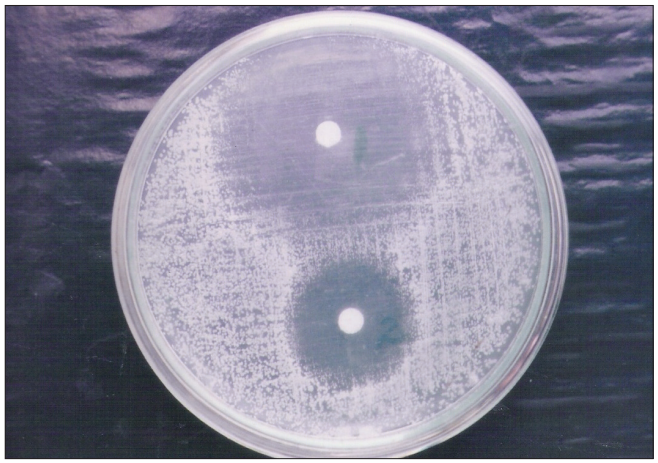

Figure 5: Fluconazole sensitive

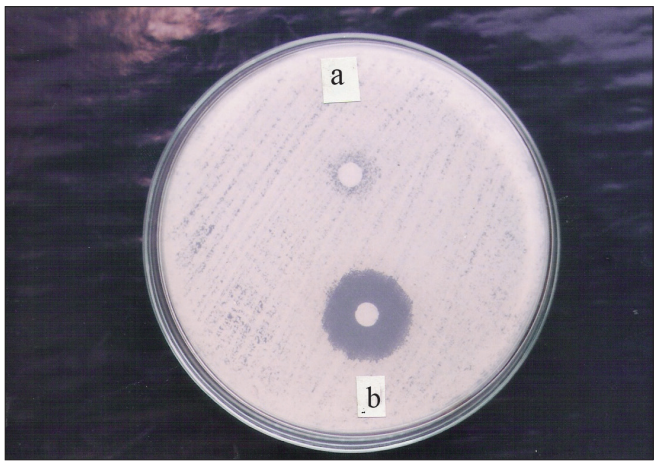

Figure 6: (a) Fluconazole resistant, (b) Fluconazole — Dose-dependent susceptible

amphotericin B, itraconazole, and clotrimazole. In the two patients with *Candida krusei*, one (50%) was sensitive to fluconazole and one (50%) was resistant to fluconazole. Both were sensitive to nystatin, amphotericin B, itraconazole, and clotrimazole.

DISCUSSION

Oral candidiasis is an opportunistic fungal infection of the oral cavity seen in HIV-infected and immunosuppressed patients. It occurs as a result of secondary infection in persons with

Table 1: Age and gender distribution

	Patient with clinical candidiasis (n = 21)			Patient without clinical candidiasis (n = 15)		
	n	Age (Mean)	Range	*n*	Age (Mean)	Range
Male	19	32.3	21 – 45	13	34.2	27 – 47
Female	2	34.5	19 – 50	2	28	22 – 44
Total	21	33.4	-	15	31.0	-

Table 2: Clinical variants of candidiasis

	Pseudomembranous candidiasis	Erythematous candidiasis
Male	14	5
Female	2	0

Table 3: Type of clinical candidiasis and $CD4^+$ cell count

	Pseudomembranous candidiasis n = 16		Erythematous candidiasis n = 5	
	n	Mean $CD4^+$ count	n	Mean $CD4^+$ count
$CD4^+ \leq 200$	4	94.5	1	99
$CD4^+ > 200$	5	417.2	4	732

Table 4: Type of candidal species identified

	Candida albicans	*Candida krusei*
Patients with clinical candidiasis	19	2
Patients without clinical candidiasis	No growth	No growth

Table 5: Clinical type and CFU/ml

Clinical type	n	CFU/ml range	Mean
Pseudomembranous candidiasis	16	200 – 43200	9175/ml
Erythematous candidiasis	5	700 – 11000	5040/ml

CFU - Colony forming units

Table 6: $CD4^+$ count and CFU/ml

$CD4^+$ count	n	Mean $CD4^+$	Mean CFU/ml
$CD4^+ \leq 200$	5	97.4	11280
$CD4^+ > 200$	9	560.2	3533

CFU - colony forming units

predisposing factors like endocrinal disorders, xerostomia, poor oral hygiene, antibiotics, and steroid therapy. Oral candidiasis is the most common oral manifestation in HIV-positive patients.[13,3,1] Oral candidiasis reflects a declining immune system and can predict the development of AIDS in HIV-positive patients.[10]

Candida albicans is the most frequently isolated fungus from the oral cavity of HIV-seropositive individuals followed by *C. stellatoidea*.[4] In this study *Candida albicans* has been isolated from 90% of the patients and *Candida krusei* in 10% of the patients. The treatment of oral candidiasis in HIV infection has been reviewed by many authors.[3] Fluconazole is an azole that acts by inhibiting lanosterol C/14 demethylase. This enzyme is essential for the synthesis of ergosterol, an important component of the fungal cell wall.[5] Polyenes damage cell membranes by binding to ergosterol and this leads to cell death.[3]

Microbiological resistance, the most common cause of refractory infection, is associated with a fungal pathogen for which an antifungal minimum inhibitory concentration (MIC) is higher than average or within the range designated as the resistant breakpoint.[9] *C. krusei* is intrinsically resistant to fluconazole.[11]

Many studies have estimated the incidence of clinical fluconazole resistance to be from: 6 – 36%, 15%, and 5–10%.[2] It has been reported that the emergence of fluconazole resistance was related to both low CD4 cell count and prolonged exposure to fluconazole,[12] and also studies have been conducted with 25 HIV-seropositive patients with clinical candidiasis and *in vitro* fluconazole-resistant candidiasis.[7] They cite immunosuppression and long-term repeated exposure to fluconazole as factors for the development of resistance.

Resistance to antifungal drugs occur due to:

- alterations in sterol biosynthesis
- alteration in the uptake of drugs
- bypass
- alteration or overproduction of target enzymes[5] sterol 14 alphademethylase (14DM), which lowers its affinity for fluconazole
- increased expression of the ERG11 gene encoding 14DM
- overexpression of genes coding for membrane transport proteins of the ABC transporter (CDR1 / CDR2) or the major facilitator (MDR1) superfamilies[8]
- switching (the ability of Candida species to generate

Table 7: Antifungals tested

Species	Fluconazole			Nystatin			Amphotericin B			Itraconazole			Clotrimazole		
	S	SDD	R	S	SDD	R	S	SDD	R	S	SDD	R	S	SDD	R
Candida albicans	14	1	4	19	-	-	19	-	-	19	-	-	19	-	-
Candida krusei	1	-	1	2	-	-	2	-	-	2	-	-	2	-	-

S - Susceptible, R - Resistant, SDD - Susceptible dose dependant

a variety of phenotypes).[9] *C. albicans* exhibits two developmental programs that provide a portion of its phenotypic plasticity, the bud-hypha transition and high-frequency phenotypic switching. Transition to a hyphal growth form provides *C. albicans* with the capacity to penetrate the tissue and disseminate, and the mutants of *C. albicans* do not form hyphae that exhibit a reduction in virulence.[6]

In conclusion Fluconazole resistance is a major problem in the treatment of oral candidiasis especially in HIV patients. Candidemia is fatal in these patients especially with a failing immune system and invasion of the fungi into the vital organs. Treating the disease aggressively becomes a priority. Therefore, it is important to perform antifungal drug susceptibility testing as part of the routine, prior to the institution of therapy, in order to avoid failure, which may lead to persistence or recurrence or dissemination of the infection. However, the sample size was small in this study and these conclusions are of a preliminary nature. Studies on a larger sample size are necessary to confirm the findings.

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