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Research Article

PREVALENCE AND ANTIFUNGAL SUSCEPTIBILITY PATTERN OF CANDIDA ALBICANS FROM LOW SOCIO-ECONOMIC GROUP

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ABSTRACT

The incidence of fungal infections has increased significantly, so contributing to morbidity and mortality. This is caused by an increase in antimicrobial resistance and the restricted number of antifungal drugs, which retain many side effects. An increasing prevalence of infections caused by newer emerging fungal pathogens has been detected in humans. *Candida albicans* is a common inhabitant of the skin, mouth, vagina and gastro intestinal tract of human beings. One of the major reasons for the increase in *Candida* infection is the development of its resistant strains to azole drugs used in the treatment of candidiasis. In this study the prevalence and antifungal susceptibility pattern of *Candida albicans* in low socio economic group has been studied in 100 urine samples. Among the 100 urine samples 26 showed positive characteristics similar to *Candida albicans*. The in vitro susceptibility testing of antifungal agents is becoming increasingly important because of the introduction of new antifungal agents and the recovery of clinical isolates that exhibit inherent or developed resistance to Amphotericin B, the Azole group of drugs during chemotherapy. Antifungal Susceptibility Testing was done for 26 *Candida albicans* isolates by Disc Diffusion Method. The organisms showed complete resistance towards Itraconazole 26 (100%), Clotrimazole 26 (100%), Nystatin 24 (92.31%), Fluconazole 21 (80.77%) and Ketoconazole 17 (65.38%) and the organisms showed highly sensitive only to Amphotericin-B 23 (88.46%). Mutations are changes to the genome of an organism. These are normally caused at low frequencies by spontaneous errors in DNA replication. The frequency of mutation is increased by mutagens, which include environmental and chemical agents such as ionizing radiation, ultraviolet light, nitrous acid and many other natural and artificial chemicals. In this study two *Candida albicans* isolates which showed sensitivity to two different antifungals were selected and subjected to UV mutagenesis and chemical mutagenesis. The results revealed that both the isolates become resistance to all the antifungals, tested against the two strains of *Candida albicans* after mutagenesis experiments by both the physical (UV) and chemical method (EMS). Today, the rapidly evolving issue of *Candida albicans* demands the obvious need for dissecting completely new regulatory mechanisms that could be targeted to control resistance. However, more studies are required to find the complete resistance mechanism responsible for their antifungal activity.

Keywords: *Candida albicans*, Prevalence, Antifungal susceptibility, Mutagenesis.

INTRODUCTION

Fungi are widespread in the environment. Some are related with animals and humans as commensals, but turn pathogenic or opportunistic after change of the host immune system [1]. Therapeutic applications of immunosuppressive drugs, the use of broad spectrum antibiotics in diverse clinical conditions and other predisposing factors are accountable for an increasing number of immunocompromised patients and consequent opportunistic infections globally. A weakened or impaired immune system provides positive conditions for pathogenic and non-pathogenic micro-organisms. In the last decades, the occurrence of fungal infections has increased dramatically due to the rise in the number of immunocompromised patients.

Candida species are the most common cause of fungal infections worldwide. *Candida* species are normal microbiota within the gastrointestinal tracts, respiratory tracts, vaginal area and the mouth and it is sexually transmitted diseases [2]. *Candida* is a yeast growth present in all females and is normally controlled by bacteria. *Candida* species differ in their antifungal susceptibility and virulence factors. The genus is composed of a heterogeneous group of organisms, and more than 17 different *Candida* species are known to be aetiological agents of human infections; however, more than 90% of invasive infections are caused by *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. krusei* [3], *C. dubliniensis*, and *C. lusitaniae*. The yeast begins to invade and colonize the body tissues by releasing powerful chemicals into the bloodstream causing such varying symptoms as lethargy, chronic diarrhea, yeast vaginitis, bladder infections, muscle and joint pain, menstrual problems, constipation and severe depression. The medical term for this overgrowth is candidiasis. Candidiasis is responsible for 90% of the cases of infectious vaginitis [4]. The most prevalent fungal pathogen of humans is *Candida albicans* which ranks as the fourth most common cause of hospital acquired infectious disease and is the primary cause of systemic candidiasis, with mortality rates approaching 50% [3a]. Prolonged usage of antifungals in treating infections caused by *C. albicans* has led to the emergence of azole resistance. This acquired azole resistance in clinical isolates of *C. albicans* mostly results in cross-resistance to many unrelated drugs, a phenomenon termed multidrug resistance (MDR) [5,6,7]. MDR is a serious complication during treatment of opportunistic fungal infections which possess grave concern given the limited number of clinically useful antifungal drugs available [8,9]. The reasons for this increase in fungal infections are multifactorial: better clinical evaluation and diagnosis, greater survival for patients with malignancies, chronic diseases, increasing number of transplants, complex surgical procedures, catheters, implants and use of wide spectrum antibiotics. *Candida* species have evolved a multitude of mechanisms to survive exposure to antifungal drugs and some of them include an over expression or mutations. Thus identification of *Candida* upto species level along with antifungal susceptibility becomes very essential. The accurate species identification of *Candida* is important for the treatment, as not all species respond to the same treatment because of the trouble of anti-fungal resistance. The aim of the present study was to know the prevalence of *Candida* spp. in lower socio-economic group, to isolate and speciate *Candida* spp. from urine specimens, to detect their antifungal susceptibility pattern.

MATERIALS AND METHODS

Study site

Total of 100 urine samples were collected from Erode district, Tamil Nadu belonging to different age groups of women ranging from 30 to 65 from January to February, 2012 using sterile uricols. All the specimens were transported immediately to the laboratory and cultured within 3 to 4 h of collection.

Isolation and characterization of *Candida albicans*

The samples were inoculated on the Sabouraud Dextrose Agar (SDA) medium and the plates were incubated at room temperature for 24 hours. After 24 h of incubation, the culture plates were examined for the appearance, size, color and morphology of the colonies were recorded. Wet mount preparation, Germ tube formation and growth on Hichrome *Candida* were carried out according to standard techniques.

Isolates that were Gram positive ovoid cells, Chlamydospores production on Corn Meal agar, green color colonies on Hichrome *Candida* differential agar medium, the germ tube production at 45°C were considered as *Candida albicans*.

Antimicrobial Susceptibility Testing

Kirby-Bauer disc diffusion method is commonly employed for antibiotic sensitivity testing [10]. Antifungal susceptibility testing was performed by NCCLS M44-A disc diffusion method [11]. Briefly, antibiotic discs containing Itraconazole (10 mcg), Ketoconazole (10 mcg), Clotrimazole (10 mcg), Fluconazole (25 mcg), Amphotericin-B (20 mcg) and Nystatin (100 units) were tested. The zones measured only that is showing complete inhibition and the diameters of the zones recorded to the nearest millimeter.

Mutagenesis

Physical Mutagenesis

For the analysis of survival rates by UV (Ultra Violet) mutagenesis, cells were grown for 24 hours at 28°C on YPD (Yeast extract Peptone Dextrose) medium plate. The cells were collected by centrifugation and suspended in sterile water and this step was performed once again. To this, 1 ml was transferred to 9 ml of sterile water (10^{-1}) and serially diluted up to 10^{-5}. From each dilution, 100µl was placed on a plates containing SDA medium, then the plates were placed under a UV lamp at a distance of 35cm and were irradiated for various periods of time. Following irradiation, the plates were incubated at 28°C. From the plates, the survival colonies were selected and again swabbed on MHA medium to determine the changes in their susceptibility pattern.

Chemical mutagenesis

For the analysis of survival rates by Ethyl Methane Sulfonate (EMS) mutagenesis, cells were inoculated into liquid YPD medium and grown for 24 hours at 28°C. The cells were washed once with sterile water, diluted and suspended in 1ml of 0.1M sodium phosphate buffer (pH-7.0). To the suspension, 2% of EMS was added. After various incubation times, the cells were washed once with 1ml of 5% sodium thiosulfate, suspended in water and spreaded on SDA plates and incubated at 28°C. The survived colonies from SDA plates were again swabbed on MHA plates to determine the changes in their susceptibility pattern.

RESULTS

The prevalence of *Candida albicans* among the low socio-economic group was assessed and recorded in table 1. Totally 100 urine samples were collected from the low socio-economic group in Erode district. Among the 100 samples 26(26%) showed positive characteristics similar to *Candida albicans*.

Table 1: Prevalence of *Candida albicans* from low socio economic group

Result	Prevalence of *Candida albicans*	Total
Positive	26 (26%)	26
Negative	74 (74%)	74
Total	100	100

These isolates were identified and confirmed based on phenotypic diagnostic methods (the colour of colonies on HICHROME agar (Fig.1), germ tube formation, Chlamydospores production on Corn meal agar medium and lack of pellicle formation on SDA broth). Table 2 also shows the phenotypic characteristics of *Candida tropicalis*, *Candida glabrata*, *Candida dubliniensis* and *Candida krusei* studied during the study period. Figure 1 shows the *Candida albicans* colonies (green) on Hichrome agar medium.

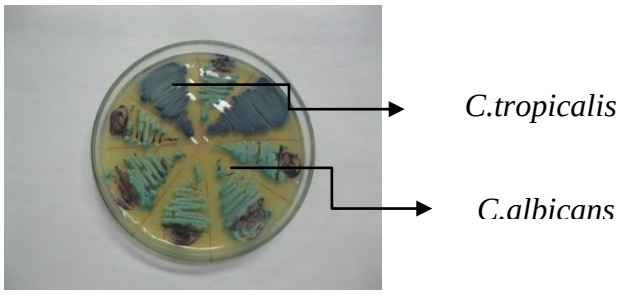


Fig. 1: Colony morphology of *Candida albicans* on Hichrome media

Table 2: Characterization of *Candida* species

S. No.	Species	Colony on Hichorme agar	Chlamydospores on Corn Meal agar	Pellicle on SDA broth	Germ tube test	Growth at 45°C
1	*Candida albicans*	Light Green	+	-	+	+
2	*Candida tropicalis*	Dark Blue	-	Small pellicle	-	-
3	*Candida glabrata*	Dark pink	-	-	-	-
4	*Candida dubliniensis*	Pale color	++	NA	++	-
5	*Candida krusei*	Pink centre with white edge	-	Thick pellicle	-	-

The *In vitro* antifungal activities of four azole derivatives (Itraconazole (IT) 10 mcg, Ketoconazole (KT) 10 mcg, Clotrimazole (CC) 10 mcg and Fluconazole (FLC) 25mcg), Amphotericin-B (AP) 100 units and Nystatin (NS) 100 units against 26 clinical isolates of *Candida albicans* are given in table 3.

Antifungal test results indicate that the yeast isolates were susceptible to Amphotericin-B 23(88.46%), Ketoconazole 9(34.62%), Fluconazole 5(19.23%) and Nystatin 2(7.69%). Various resistant levels were detected against other antifungal drugs but to Itraconazole and Clotrimazole all the isolates of *Candida albicans* were found to be 100% resistant. Two isolates of *Candida albicans* showing higher resistance patterns were selected and subjected to the physical (Ultra violet radiation (UV)) and chemical mutagenesis (Ethyl methyl sulfonate (EMS)). Table 4 shows the surveillance rate of the isolates after mutagenesis. Even after the exposure of *Candida albicans* to physical and chemical mutagens they produced the colonies of growth on Muller-Hinton agar.

The UV and EMS exposed isolates of *Candida albicans* were tested with the same antifungal agents which are tested against the isolates before mutagenesis.

Table 3: Susceptibility pattern of the isolates

S. No.	Antifungal agents	Disc potency	Sensitive isolates no (%)	Resistant Isolates no (%)
1	Itraconazole	10 mcg	-	26 (100)
2	Ketoconazole	10 mcg	9 (34.62)	17 (65.38)
3	Clotrimazole	10 mcg	-	26 (100)
4	Fluconazole	25 mcg	5 (19.23)	21 (80.77)
5	Amphotericin-B	20 mcg	23 (88.46)	2 (7.69)
6	Nystatin	100 units	2 (7.69)	24 (92.31)

Table 5 shows the antifungal susceptibility pattern of the isolates before and after mutagenesis. Before mutagenesis the isolate A1 of *Candida albicans* showed the sensitivity pattern against Amphotericin-B and intermediate sensitivity against Ketoconazole and Fluconazole and B1 showed sensitivity against Amphotericin-B and Nystatin. But after mutagenesis both A1 and B8 isolates of *Candida albicans* become completely resistant to the antifungal drug tested (Fig.2). The resistance of the isolates may be acquired due to the exposure of colonies to the physical and chemical mutagens.

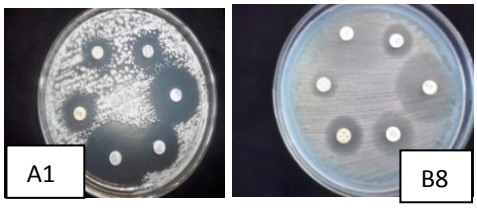
Table 4: Surveillance rate of the isolates after mutagenesis

S. No	Strain no	Time of Exposure	Growth after mutagenesis	
			UV Mutagenesis	EMS mutagenesis
1	A1	10 sec	+	+
		20 sec	+	+
		30 sec	+	+
2	B8	10 sec	+	+
		20 sec	+	+
		30 sec	+	+

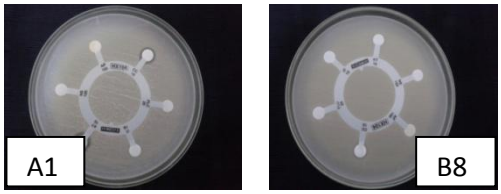
Table 5: Antifungal susceptibility pattern of the isolates before and after mutagenesis

Antifungal susceptibility pattern before mutagenesis							
S. No	**Strain no**	**IT**	**KT**	**CC**	**FLC**	**AP**	**NS**
1	A1	12 (R)	22 (IS)	13 (R)	30 (IS)	16 (S)	13 (R)
2	B8	- (R)	11 (R)	- (R)	- (R)	30 (S)	28 (S)
Antifungal susceptibility pattern after mutagenesis							
1	A1	UV	- (R)	- (R)	- (R)	- (R)	- (R)
		EMS	- (R)	10 (R)	- (R)	- (R)	- (R)
2	B8	UV	- (R)	- (R)	- (R)	- (R)	- (R)
		EMS	- (R)	10 (R)	- (R)	- (R)	- (R)

Before mutagenesis



After mutagenesis (UV)



Chemical mutagenesis (EMS)

Fig.2: Antifungal susceptibility pattern of the isolates before and after mutagenesis

DISCUSSION

Candida albicans is the predominant cause of invasive fungal infections [12] and represents a serious public health challenge with increasing medical and economic importance due to the high mortality rates and increased costs of care and duration of hospitalization [13,14]. Several *Candida* species are commensal and colonize the skin and mucosal surfaces of humans. Critically ill or otherwise immunocompromised patients are more prone to develop both superficial and life-threatening *Candida* infections [15].

Candida albicans prefers the presence of moist areas of the body, which is why it is usually found in the genital and oral regions. Many other microorganisms grow in healthy mucus membranes in the body. However, when other organisms become depleted, the yeasts are able to out compete for the limited resources. When the yeast takes over in the mouth or throat, it is often called 'thrush' and causes a sore throat along with a white coating in the mouth.

When the infection is in the female genital regions, it is called vulvovaginitis and can cause extreme itching and burning sensations along with possible white discharge. An infection caused by *Candida* is termed candidiasis or candidosis. Mycoses caused by these fungi show a wide spectrum of clinical presentations and can be classified as superficial, as with cutaneous and mucosal infections, to deep, widespread and of high severity, as is the case with invasive candidiasis. Although this commensal organism normally colonizes mucosal surfaces in an asymptomatic manner, it can become one of the most significant causes of a disabling and lethal infection [16, 17]. In the early 1980s, fungi emerged as major causes of nosocomial infections, mainly affecting immunocompromised patients or those who were hospitalized for long periods due to serious underlying diseases [18].

Candida species belonging to the microbiota of healthy individuals can be found scattered in the environment. It is believed that most people usually have a single strain of *Candida* in different places in the body for a long period. However, some individuals have more than one strain or species at the same time, and this is commonly observed among hospitalized patients [19]. Moreover, the potential for *Candida* species to become pathogenic should be appreciated. A crucial component of this versatility is the fact that these organisms survive as commensals in diverse and distinct anatomical sites, each with its particular environmental stresses. Although *Candida* species can infect different anatomical sites of the human host, evidence exists that immune protection is site-specific for each type. Moreover, cutaneous candidiasis and vaginal infections are more likely to be associated with a phagocytic response involving neutrophils and mononuclear phagocytes [18]. The urinary tract is the anatomical site most conducive to the development of infections in hospitalized patients, although this remains a problem of questionable significance [20, 18].

In the present study the prevalence and its antifungal susceptibility of *Candida albicans* isolated from urine sample among the low socio-

economic group was assessed and reported. Totally 100 urine samples were collected from the low socio-economic group in Erode district. There are several features essential for in vitro morphological transition of *C. albicans*. The earliest workers with *C. albicans* found that changes in the growth environment of *C. albicans* led to changes in cell shape. However, no one has ever been able to discover any environmental "morphogen" that acts alone and under all conditions to provoke the growth of cells from any *C. albicans* isolate in only one morphological form. Some environmental factors that favour the filamentation in *C. albicans* are: temperature of 37–40°C, pH around 7.0 and an initial blastospore concentration not exceeding 106/ml. Also, certain chemicals, such as N-acetyl-D-glucosamine, amino acids, biotin, sulfhydryl compounds, heme group, zinc, and serum, etc. are required. The production of germ-tubes in serum has remained the method of choice for identifying *C. albicans* in clinical specimens. Although serum is excellent at promoting the yeast to mycelial conversion, unfortunately its chemical complexity makes it unsuitable for studying the molecular basis for inducing the hyphal form. In the present study among the 100 urine samples 26(26%) showed positive characteristics similar to *Candida albicans*. These isolates were identified and confirmed based on phenotypic diagnostic methods (the colour of colonies on HICHROME agar, germ tube formation, Chlamydospores production on Corn meal agar medium and lack of pellicle formation on SDA broth). In the current study along with *C.albicans*, *Candida tropicalis*, *Candida glabrata*, *Candida dubliniensis* and *Candida krusei* were also isolated from urine. In Chile, the prevalence of *C. albicans* has changed, and a progressive increase of non-albicans infection has been observed; *C. parapsilosis* was the most frequent species, followed by *C. tropicalis* and *C. glabrata*. According to the Brazilian Network Candidaemia Study, *C. albicans* accounted for 40.9% of cases in Brazil, followed by *C. tropicalis* (20.9 %), *C. parapsilosis* (20.5 %) and *C. glabrata* (4.9 %) [21,22]. In a study in United States which conducted by Richter, 593 cases of vaginal yeasts, including *C. albicans* (420 cases), *C. glabrata* (112 cases), *C. parapsilosis* (30 cases), *C. krusei* (12 cases), *Saccharomycess cerevisiae* (9 cases), *C.tropicalis* (8 cases), *C. lusitaniae* (1 cases) and *Trichosporon* (1 cases) were isolated [23]. In a study conducted in Turkey in 2000 on clinical isolated *Candida* from vagina, the most frequent species were *C. albicans* (50%), *C.glabrata* (26.92 %), *C. krusei* (11.53 %), *C. kefyr* (8.97 %), *C. tropicalis* (1.28 %) and *C. parapsilosis* (1.28 %), respectively [24]. In a study in Belgium in 2002, *C. albicans* was recognized as the most frequent pathogen with a frequency of 68.3 %, followed by *C. glabrata* and *C. parapsilosis* [25]. In another study in the United States in 2005 which conducted by Trama et al., 1316 positive cultures including *C. albicans* (80.2 %), of *C. glabrata* (14.3 %), of *C. parapsilosis* (5.9 %) and *C. tropicalis* (8%) were recognized [26]. According to another study in Bangladesh in 2007 from 172 RVVC patients 125 cases (72.7 %) were *C. albicans*, 29 cases (16.9 %) were *C. glabrata*, 13 cases (7.5 %) were *C. tropicalis*, and about 5 cases (2.9 %) were *C. krusei* [27]. In 2009 a study was conducted on 1050 women, from 215 studied VVC patients in this study, the most frequent species were *C. albicans* (46.9 %), *C.glabrata* (36.7 %), *C. parapsilosis* (10.2 %), *C. tropicalis* (2.8 %), *C. Kefyr* (1.9 %) and *C. krusei* (1.4 %) [28]. Based on a study conducted by Aqhamirian in Iran in 1386 on 128 women suffered from vaginal illnesses, *C.albicans* cover 83 % of isolated fungal species, the 17% remaining cases include *C. glabrata*, *C. tropicalis*, *C.parapsilosis,* and *C. krusei* [29]. From the past studies by other authors and the current study also reveals the same result that *C.albicans* was the predominant strains noted in all the studies. Candiduria is a common nosocomial infection, which involves the urinary tract system as asymptomatic candiduria. The disease is most commonly caused by *C.albicans* [30]. In the current work 26 isolates of *C.albicans* was isolated from the urine sample of females belonging to low socio-economic group. In previous studies they isolated 22% of *Candida* species in urine samples from patients admitted to intensive care units [31].

Data from previous studies suggest that prolonged or intermittent treatment with antifungal agent modifies *C.albicans* prevalence, increasing the frequency of other *candida* species. Acquired resistance has been associated with mutations in the target gene leading to lower affinity of the azole compound to the enzyme, upregulation of the enzyme level or by active transport of the azole out of the cell mediated by efflux pumps. Antifungal test results of the present study indicate that the *C.albicans* were susceptible to Amphotericin-B 23(88.46%), Ketoconazole 9(34.62%), Fluconazole 5(19.23%) and Nystatin 2(7.69%). Various resistant levels were detected against other antifungal drugs but to Itraconazole and Clotrimazole all the isolates of *Candida albicans* were found to be 100% resistant. In previous work they described about the conventional antifungal drugs and their cellular targets [32]. Among the azole chemical class described Ketoconazole, Fluconazole, Itraconazole target against Ergosterol synthesis, Amphotericin-B and Nystatin belonging to polyenes chemical class target against the Ergosterol (membrane function). Resistance may involve selected azoles or several azoles depending on the underlying mechanism and the various mechanisms may act alone or in concert [33, 34]. Although azole resistance has been described in invasive isolates, most resistant isolates have been detected after long-term treatment of mucosal infections. Overall, azole resistance in isolates belonging to normally susceptible species is still an infrequent event despite their use for several decades and nowadays for prophylaxis, empirical and preventative therapy as well as for the management of proven disease [35, 36]. However, a recent study reported reduced fluconazole susceptibility in 19% of 243 candidaemia cases including in 8% *C. albicans*, 4% *C. tropicalis* and 4% *C. parapsilosis* [37].

Mutations are changes to the genome of an organism. These are normally caused at low frequencies by spontaneous errors in DNA replication. The frequency of mutation is increased by mutagens, which include environmental and chemical agents such as ionizing radiation, UV irradiation, methyl-nitro-nitrosoguanidine, ethylmethane-sulfonate, nitrous acid and many other natural and artificial chemicals. Induced mutations can include changes not only to the nucleotide sequence, but also the structure and function of the DNA such as the creation of pyrimidine dimers and hydroxyl radicals. Different mutagenic agents have been used to obtain *C.albicans* mutants. In the current work two *Candida albicans* isolates which showed sensitivity to two different antifungals were selected and subjected to UV mutagenesis and chemical mutagenesis. The results revealed that both the isolates become resistance to all the antifungals, tested against the two strains of *Candida albicans* after mutagenesis experiments by both the physical (UV) and chemical method (EMS). The resistance of the isolates may be acquired due to the exposure of colonies to the physical and chemical mutagens.

CONCLUSIONS

Today, the rapidly evolving issue of resistance demands the obvious need for dissecting completely new regulatory mechanisms that could be targeted to control resistance. Novel mechanisms described earlier for pathogenic fungi clearly hold promise that can facilitate the development of better antifungal strategies to efficiently control the human fungal diseases. Elucidating these mechanisms may provide new foundations for antifungal chemotherapy and can present an exciting challenge for the future investigation.

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CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

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