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New Emerging Resistant Strains of *Candida albicans* Against Known Antifungal Drugs

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***Candida albicans*, Antifungal resistance, Germ tube test**

ABSTRACT

Candida albicans, an opportunistic fungus, causing life-threatening infections in humans. Recently, there has been a profound increase in the prevalence of fungal resistance against antifungal drugs. Therefore, *C. albicans* isolated from clinical samples were investigated against a variety of antifungal drugs. Fifteen pus and urine samples from diagnostic laboratories were collected and cultured on Saboraud dextrose agar under standard conditions. These cultures were identified by Germ Tube Test, then cultured on selective Brilliance™ Candida agar media. Out of 15 samples, eight contained *C. albicans*. These strains were subjected to susceptibility testing against fluconazole, ketoconazole, itraconazole, voriconazole, amphotericin B, and caspofungin. It was observed that 3 strains of *C. albicans* (AZM1, AZM2, and AMZ3) showed significant resistance to all drugs under investigation, while AZM4 and AZM5 strains exhibited moderate resistance while others were sensitive. Molecular 18S rDNA sequencing analysis of resistant fungal strains was performed using NS1 5' and NS24 primers and a phylogenetic tree was constructed. The results indicate that *C. albicans* is currently an emerging problem. So, new therapeutic tools are required to improve the prognosis of the disease.

INTRODUCTION

Prevalence to antimicrobial resistance has increased over the years and has been reported in different regions worldwide including developing and underdeveloped countries (Aceng *et al*., 2005; Eliopoulos *et al*., 2002). The reasons for the emergence of resistance may involve excessive use of drugs, prolonged hospitalization, irrational treatment strategies, and new emerging strains of pathogenic microbes. The frequency of fungal infections is on a rise and there is an utmost need to characterize these strains to ensure effective treatment (Fridkin and Jarvis, 1996).

Among many species of genus *Candida,* there are many diverse microorganisms but *Candida albicans* is seen in both commensal and pathogenic states. There has been increased concern about *C. albicans* infections over the past few years. About 80% of all the isolates belong to this candidal species (Raju and Rajappa, 2011). Identification of this species is of extreme importance, as different species of *Candida* differ in their infection pattern and susceptibility to different antifungal agents (Scully *et al*., 1994).

C. albicans is the most prevalent human pathogen causing superficial and fetal systemic infections (Soysa *et al*., 2008). Most of the emerging fungal infections arise as breakthrough infections in patients treated with various antifungal therapeutic agents. The contributing pathogens, prevalence rate, and severity of infection depend upon the basic mechanisms and underlying conditions. The mortality rate varies and has not been reported in many cases and currently, the fungal infection is regarded as a potential threat to global health (Pfaller *et al*., 1998; Kibbler *et al*., 2003). It is not easy to treat these infections as a variety of strains affects different tissues and organs, resistance, and scarcity of new antifungal drugs. The

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differences in fungal species showing different intrinsic antifungal susceptibilities reflect genetic variations that usually occur during their reproduction cycle (De Queiroz, 2007; Taylor *et al*., 2000).

It has been observed that *C. albicans* strains are becoming resistant to amphotericin B, fluconazole, polyenes, and 5-flucytosine (Goldman *et al*., 2004). The differences in resistance mechanisms have been observed in the molecular analysis (Kanafani and Perfect, 2008; Selmecki *et al*., 2006). From an evolutionary point of view, none of the resistance mechanism works alone.

The purpose of this study was to find and identify the emerging strains of *C. albicans* to avoid treatment and prognosis failures. We analyzed 15 clinical isolates collected from pus and urine samples. From these isolates, an antimicrobial susceptibility assay was performed against *C. albicans* using amphotericin B, caspofungin, fluconazole, ketoconazole, itraconazole, and voriconazole. Molecular identification and evolutionary relationship among resistant strains of *C. albicans* were studied.

MATERIALS AND METHODS

Sample collection and isolation

A total of 15 samples (pus and urine) were collected from various diagnostic labs. The sabouraud dextrose agar (SDA) medium plates were prepared (Goldschmidt *et al*., 1991) for culturing purposes. The samples were streaked onto the medium by using the standard streak plate method. Plates were incubated at 25°C for 48 h (Fromtling and Bulmer, 1978).

Identification of Candida albicans

Germ tube test (GTT) based on germ tube formation is a rapid and cost-effective screening test which is being used for years for the persuasive identification of *C. albicans* (Lee *et al*., 1999). The formation of germ tube in *C. albicans* is one of its major virulence factors. It is positive for *C. albicans* and *C. dubliniensis*. The bud formation was analyzed under a microscope to identify the *C. albicans*. The pure streaked culture was transferred onto selective Brilliance™ Candida Agar Base media (CHROM agar Candida Oxoid, CM1002A). The petri plates were placed in an incubator at 37°C and the typical color appearance of microorganisms was observed. *C. albicans* appear green in color on this medium while *C. tropicalis* indicate metallic blue color and other fungi appear white to mauve (Prato *et al*., 2008).

Antifungal susceptibility assay

Six anti-fungal drugs including amphotericin B, ketoconazole, voriconazole, caspofungin, itraconazole, and fluconazole were used. These drugs were prepared by serial dilution (Lalitha, 2004). Disk-diffusion testing was employed to evaluate the susceptibility of *C. albicans* against antimicrobial drugs. Filter paper discs impregnated with drugs to be tested were used. A pure culture of *C. albicans* was taken and emulsified in normal saline; it was mixed thoroughly so that no solid material from the colony was visible. A sterile culture swab was dipped in the emulsion; extra fluid was removed by gently squeezing the swab inside of the tube. With a culture swab, a lawn of growth was streaked onto the media agar plates. Sterilized forceps were used to place the individual antifungal disks on the streaked plate. The plates were inverted for 24 h at 25-30°C. The drug diffuses from the disc into the agar and the strains that were susceptible to the impregnated drug showed a zone of inhibition (Heatley, 1944). Zone of inhibition (if present) was measured, and then compared with the table of standards of each disk to decide either the strain was resistant or sensitive to that antifungal drug. The results of antifungal susceptibility testing were recorded as sensitive (S), resistant (R), and intermediate (I) based on the diameter of the zone of inhibition (Heatley, 1944).

Extraction of fungal DNA

The genomic DNA from the resistant strains of *C. albicans* (AZM1, AZM2, and AMZ3) was extracted using a commercial DNA extraction kit as per the manufacturer's instructions (Promega, USA). The DNA was quantified by spectrophotometer at OD 260/280 nm by ratios 1.5-1.6. The sensitivity of the PFPRIM-F3 and PFPRIM-R4 primers was calculated by amplification through PCR for sequentially diluted concentrations (10ng-100ng) (Ilkit and Guzel, 2011).

Molecular sequencing and construction of a phylogenetic tree

The PCR reaction for the detection of the 18S rDNA gene of *C. albicans* was carried out. For this purpose, 25µl of reaction mixture containing 5.5µl of nuclease-free water, 12.5µl of Master mix (2X Green GoTaq), 2.5µl of 20 pmol PFPRIM-F3 primer and 2.5µl of 20 pmol PFPRIMR4 primer, and 2µl sample of genomic DNA was prepared. PCR was performed in the thermal cycler (Applied Biosystem 9902, Singapore) according to the PCR program (Embong *et al*., 2008). The analysis of the PCR product of the 18S rRNA gene of *C. albicans* was carried out on 1% agarose gel. 18S rDNA region was analyzed and sequenced by using primers NS1 5' (GTA GTC ATA TGC TTG TCT C) 3' and NS24 5' (AAA CCT TGT TAC GAC TTT TA) 3'. PCR reaction was carried out by taking 20ng of genomic DNA and used as a template in 30µl reaction mixture using an *EF-Taq* (SolGent,

Korea). A basic local alignment search tool (BLAST) at NCBI was applied to align the 18S rRNA sequences of *C. albicans* (AZM1, AZM2, and AMZ3) with known and related sequences in the database, and the percentage of homology was checked. *C. albicans* sequences with >99% similarity was considered to be of the same phylotype (Felsenstein, 2008; Saitou and Nei, 1987). Phylogenetic tree was constructed with the help of neighbor-joining method using custal omega online server (http://www.ebi.ac.uk/Tools/msa/clustalo/).

RESULTS

From 15 clinical specimens (Table I), eight *C. albicans* were isolated (7 urine and 1 sample of sputum). The germ tube test was positive which confirms the presence of *C. albicans* in these samples. The germ tube formation has been shown in the form of bud (Fig. 1A). A short hyphal (filamentous) extension arising laterally from yeast cells, with no constriction at the point of origin was observed microscopically. Germ tube was half the width and 3 to 4 times the length of the yeast cell and there was no presence of a nucleus. Brilliance™ Candida Agar Base, a selective medium was used for the isolation of *C. albicans* from other candidal and yeast species. In this media, *C. albicans* colonies appeared green in color which confirms the purified culture of this species as compared to other *Candida* species (Fig. 1B).

Table I. Collection of samples from diagnostic laboratories.

Sample code	Positive for *C. albicans*
Sputum	
S1	-
S2	+
S3	-
Urine	
U1	+
U2	+
U3	+
U4	-
U5	+
U6	+
U7	+
U8	-
U9	-
U 10	-
U 11	-
U 12	+

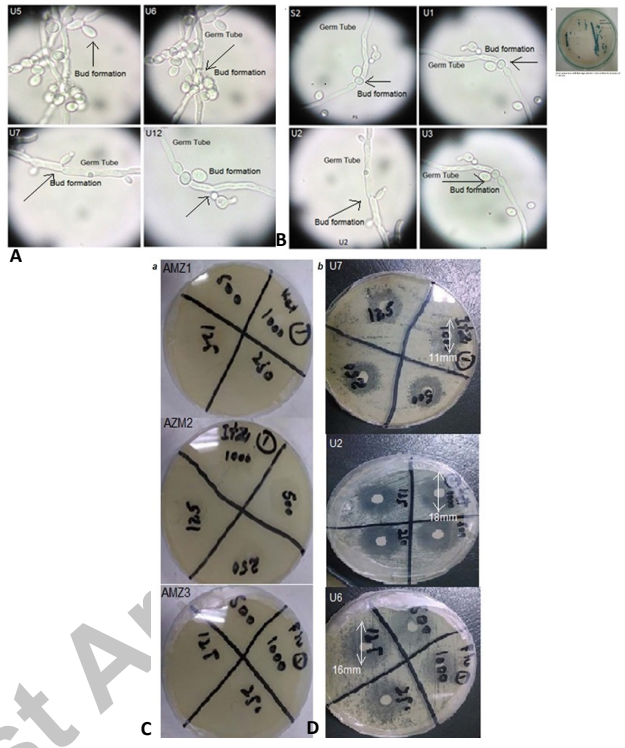

Fig. 1. *Candida albicans*: A, B shows positive germ tube test of *C. albicans* (S2, U6) showing bud formation and germ tube extension. C in S2: (Sputum) and U6 (urine samples). C shows resistance of *C. albicans* (AZM2) tested antifungal drugs. D shows zone of inhibition (U2) against tested drugs.

By disc diffusion and minimum inhibitory concentration method, we studied the susceptibility of eight identified strains of *C. albicans* against six antifungal drugs. From these 8 cultures, five candidal strains (AZM1-5) showed resistance to these drugs. Strain AZM1, AZM2, and AMZ3 of *C. albicans* showed significant resistance against all tested antifungal drugs (Fig. 1C) even at 1000 µg/ml as compared to AZM4 and AZM5 which were sensitive to amphotericin B, voriconazole, and caspofungin (Table II). Strain U-2, U-6, and U-7 were susceptible to most of these drugs even at 125 µg/ml (Fig. 1D).

The 18S rDNA region of resistant strains AZM1, AZM2, and AMZ3 of *C. albicans* was characterized to observe the novelty. The PCR product of 18S rDNA has a 600-320 bp size. According to blast outcome at NCBI server, it was confirmed that *C. albicans* strains AZM1, AZM2, and AMZ3 showed 100% sequence identity with our Candidal strains at the NCBI database. Phylogenetic trees were constructed by the neighbor-joining method and the evolutionary relationship was analyzed (Fig. 2). We have observed that strains AZM1, AZM2, and AMZ3 of *C.*

albicans are evolutionarily related with a partial sequence of *C. albicans* with accession numbers: KX557291, KX557292, and KX557293, respectively when a blast on NCBI database.

Table II. Antibiotic susceptibility of *C. albicans* against selected anti-fungal drugs.

Antibiotic durg	U-1	S-2	U-3	U-5	U-12	U 2	U 6	U-7
Ketoconazole (KT) (µg/ml)								
125	R	R	R	R	R	R	S	R
250	R	R	R	R	I	I	I	I
500	R	I	R	I	S	S	S	S
1000	R	I	R	S	S	S	S	S
Amphotericin B (AB) (µg/ml)								
125	R	R	R	R	R	S	R	R
250	R	R	R	I	I	S	S	R
500	R	R	R	I	S	S	S	S
1000	R	I	R	S	S	S	S	S
Voriconazole (VRC) (µg/ml)								
125	R	R	R	S	R	R	R	R
250	R	R	R	S	R	R	R	R
500	R	R	R	S	R	R	I	I
1000	R	R	R	S	I	I	I	S
Caspofungin (CAS) (µg/ml)								
125	R	R	R	I	R	I	R	I
250	R	R	R	I	R	S	I	I
500	R	R	R	S	R	S	S	S
1000	I	R	R	S	R	S	S	S
Itraconazole (IT) (µg/ml)								
125	R	R	R	R	R	R	R	S
250	R	R	R	R	R	R	R	S
500	R	R	R	R	R	S	R	S
1000	R	R	R	S	I	S	I	S
Fluconazole (FLC) (µg/ml)								
125	R	R	R	R	R	S	S	I
250	R	R	R	R	I	S	S	S
500	R	I	R	I	I	S	S	S
1000	I	I	R	S	I	S	S	S

*Indications: R, resistance (<12mm); I, intermediate (13-17mm); S, sensitivity (>17mm).

DISCUSSION

The antifungal drug resistance is recognized as a threat to public health due to emerging strains, comparatively less awareness, and research. This study highlights the need for improvement in antifungal therapy to either stop or minimizes the emergence of resistance. The activities of six antifungal drugs including amphotericin B, caspofungin, itraconazole, voriconazole, fluconazole, and ketoconazole against *C. albicans* were analyzed. Van *et al.* (1989) conducted the antifungal assay using amphotericin B, itraconazole, and fluconazole against systemic *C.* albicans (Van *et al*., 1989). The antifungal assay showed the resistance of *C. albicans* against the azole class of compounds (Sanglard *et al*., 2003). Such studies contained a limited number of samples and a susceptibility test was performed only against azole drugs. A total of 8 isolates were confirmed as *C. albicans* among which five strains were resistant to antifungal drugs. *C. albicans* was primarily isolated by culturing on Sabouraud dextrose agar (SDA) and subsequently cultured on selective Brilliance™ Candida Agar Base, however, conventional techniques of identification are time-consuming and are based on extensive series of tests like carbohydrate fermentation and assimilation. While in other studies, Candida species were distinguished by using Pagano-Levin agar, which works on the reduction of triphenyl tetrazolium chloride. This medium produces colonies of *C. albicans* having pale color, while colonies of other *Candida* species show variable degrees of pink coloration. The sensitivity of Pagano-Levin agar is similar to SDA, but SDA has an advantage due to ease of availability and is standard media against fungal species (Samaranayake *et al*., 1987). Morphologically, *C. albicans* was identified by germ tube test (Lee *et al*., 1999).

Antifungal susceptibility was performed by the disk diffusion method (Jorgensen and Turnidge, 2015). It has been demonstrated that the disc diffusion method is more useful as compared to other antifungal susceptibility techniques (Serrano *et al*., 2004; Valle *et al*., 2004). Strain AZM1, AZM2, and AMZ3 of *C. albicans* showed significant resistance against all tested antifungal drugs, however, AMZ4 and AMZ5 showed resistance to ketoconazole and itraconazole only while the rest of the strains were susceptible. The prevalence and antifungal susceptibility of *C. albicans* showed that isolates were susceptible to amphotericin B followed by 50 % of resistance to fluconazole only (Ajenjo *et al*., 2011). We have observed that strain AZM1, AZM2, and AMZ3 of *C. albicans* exhibited resistance to voriconazole which was found effective drug against this fungus in previous studies (Krcmery and Barnes, 2002). The resistant strains of *C. albicans* were characterized by molecular 18 rDNA technique using NS1-NS24 primers (Aubert *et al*., 1996).

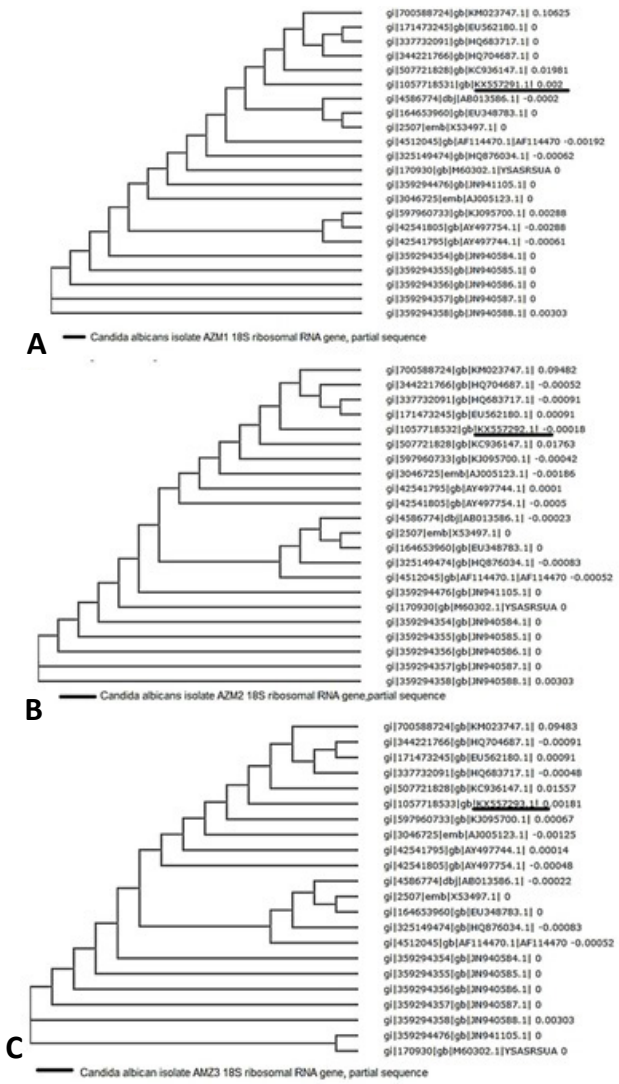

Fig. 2. Phylogenetic tree indicating the evolutionary relationship of resistant strains. These were constructed by Neighbor Joining method using Clustal Omega server. (A) *C. albicans* strain AZM1 (U-1), (B) C. *albicans* strain AZM2 (S-2), (C) *C. albicans* strain AMZ3 (U-3).

CONCLUSION

We identified new emerging strains of *C. albicans* that are resistant to available antifungal drugs. This is an alarming situation, as in most cases of antifungal therapies, the azole class of compounds is being used as a drug of choice. There is a need for developing and designing of new drug molecules to cure this pathogenic organism and this study would be helpful to improve prognosis.

DECLARATIONS

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IRB approval

The study has been approved by the Institutional Ethical Committee of Institute of Molecular Biology and Biotechnology, Bahauddin Zakariya University Multan, Pakistan under notification no. IMBB-05/17.

Ethical statement

The present study was ethically approved by the Institute of Molecular Biology and Biotechnology, Bahauddin Zakariya University, Multan, Pakistan.

Statement of conflict of interest

The authors have declared no conflict of interest.

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