

Evaluation of Caspofungin Susceptibility by Microdilution Broth in *Candida albicans* Clinical Isolates in Iran

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In recent years, opportunistic and systemic infections resulting from *Candida albicans* have increased significantly. This organism has an instable resistance to common antifungal medicines, which is mainly because of fluctuations in the structural and regulatory genes of the fungus. The present study aims to investigate the antifungal effects of caspofungin on clinical strains of *Candida albicans* as well as review the medical resistance pattern. In the present study, 52 samples of mucosal, cutaneous and visceral lesions of patients with suspected candidiasis. Different tests were taken to identify *Candida albicans* yeast. The MIC amounts of the medicine were also measured using RPMI1640 and broth micro-dilution method. The gene regions of samples were enhanced by 514 bp using PCR method. In the test of medical sensitivity, MIC fluctuations between 0.03 and 8 µg/ml were studied, of which 85% of strains were found to be in MIC<0.5-2 range and 15% were in MIC range of 4-8 µg/ml.

Key words: *Candida albicans*, Caspofungin, FKS1 Gene, Medical Resistance Pattern.

Increased occurrence of fungal infections like candidiasis, as well as their epidemiological changes and medical resistances highly justify the need to conduct studies on fungal infections. Among all *Candida* species, *Candida albicans* is regarded as the most pathogen and the most common strain which has become more important due to its resistance toward anti-fungal medicines. This yeast forms part of the natural flora of mucus and skin and is restricted to skin in form of saprophyte. In favorable occasions or when the body is weak, or instances wherein more systemic corticosteroids and antibiotics are taken and also in malignancies, the yeast can turn into pathogen and can result in mucosal or cutaneous infections, and in some uncommon cases it may cause systemic diseases¹⁻⁴.

Candida albicans is one of the known opportunistic organisms among the second to fourth agents separated from the blood of patients hospitalized in AIDS and cancer care centers. As too many attempts are made in prophylaxis and treatment of these diseases and also that the disease agent is eukaryotic, the applied medicine usually leave irreversible adverse effects on different organs of patients' bodies such as kidneys or liver, which will again add to the severity of the disease^{5,6}.

Today different medicines including azole family, polyene and echinocandins like caspofungin are used in treating such infections. The resistance of *Candida* species to anti-fungal medicine is seen in various studies, each getting resistant to the medicine through a different mechanism^{7,8}.

Echinocandins are a new class of anti-fungal medicine with a vast range of operation. They prevent 1&3 β-D-glucan synthase enzyme from operating which is needed for forming the

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1&3 β -D-glucan polymer existing in the fungal cell walls^{9,10}.

Caspofungin is the first anti-fungal medicine in this class which is imported to Iran and used at very expensive prices. As echinocandins like caspofungin are a new class of anti-fungal medicine in the recent decade, and as cases of resistance of *Candida* to this antibiotic and consequently the failure in treatment with these medicines have been reported despite their vast usage, studying the development of medical resistance to this medicine seems vital. Meanwhile, incorporating the medical resistance patterns and molecule methods used for diagnosing the resistant *Candida* strains and the mechanisms of their resistance seem to be proper solutions.

In the present study, after the MIC was determined through broth micro-dilution method, and upon using the RPMI 1640 culture medium and adjusting them with the standard CLSI tables, the resistant strains were isolated through PCR. This method proved to be quick and reliable.

The present study aims to investigate the resistance of *Candida albicans* isolates taken from patients with cutaneous, mucosal and visceral candidiasis, since the answer to these question is important in treating these types of treatment-resistant infections.

MATERIALS AND METHODS

Strains and Combinations

In the present study, 52 clinical samples were studied. Samples were taken randomly from patients referring to four hospitals in the city of Gorgan (northern part of Iran), and Tehran (central region of Iran) as well as the collection of samples belonging to Tarbiat Modares University. In order to identify *Candida albicans* strains, a direct microscopic study was first conducted and samples were inseminated in Sabouraud dextrose agar medium (Merck Co.). Then, studies were conducted on production of chlamyds spores in the CMA-T80 medium and how they were cultured on chromogenic Chromagar medium. Glucose absorption tests were also taken by API120 kit, and the yeast's ability to grow in temperatures of 37, 42, and 45 centigrade degrees were also studied. Lastly, an exclusive PCR study was conducted to differentiate it from dubliniensis species.

Sensitivity-Determination Test

For determining the medical sensitivity and MIC of the isolates which were diagnosed and confirmed as *Candida albicans*, the standard method of broth micro-dilution was applied in accordance with what has been presented in the CLSI-M27A. In 2007, this committee proposed an instruction for determining the MIC and evaluating the medical sensitivity of echinocandins in *Candidas*, according to which strains with MIC \leq 2 μ g/ml are considered sensitive to the medicine and those with MIC $>$ 2 μ g/ml are considered non-sensitive. Using the term "non-sensitive" instead of "resistant" is due to lack of cases of *Candidas*' resistance to echinocandins at that time¹¹⁻¹³. In the present study, *Candida albicans* ATCC90028 has been used as the control strain in the sensitivity test.

In order to determine the sensitivity of *Candida albicans* strains against caspofungin, first a suspension of the 48-hour cultures of the above mentioned strains grown in Sabouraud dextrose agar medium plus distilled water was prepared, and then the initial sample containing 10⁶ yeasts as per each cc of the distilled water was prepared by spectrophotometer in the wavelength of 530 nanometers and with transition of 75-77 percent. After that, samples were diluted first at the ratio of 1:10 with distilled water and finally at the ratio of 1:100 with RPMI1640 culture medium so that the final number of yeasts reach 10³cfu/ml.

For preparing the stock caspofungin dilution, 0.8 grams of pure caspofungin powder (prepared by Switzerland's Basel Co.) was dissolved in dimethyl sulfoxide (DMSO), and RPMI1640 culture medium (with glutamines, without bicarbonate, and with PH indicator of Sigma Co.) and MOPS buffer (Sigma Co.) were used for diluting it.

In order to conduct broth micro-dilution test, 9 dilutions of 0.03-8 micrograms per milliliters range were prepared. After preparing a serial dilution from caspofungin and inseminating yeasts to the micro plates, they were incubated under the temperature of 35 centigrade degrees for 48 hours. A combination of RPMI and yeast were used as the positive control in one well, and a combination of RPMI and medicine were used as the negative control. According to the standard CLSI tables, those strains with MIC $>$ 2 micrograms per milliliter

were considered as non-sensitive.

DNA Extraction, Amplification by PCR Method

After resistant strains were determined, their DNA were extracted by glass bead/phenol-chloroform method. In this method, after lubricating the yeast with a lubricating buffer (10mMTris, 1mMEDTAPH:8, 1%SDS, 2%TritonX-100, 100mM NaCl), phenol-chloroform and glass bead with 0.5 mm diameter were used to completely destroy centrifuge wall at 10000 rpm in 5 minutes, and then they were washed with ethanol 70% and were suspended in TE buffer.

Reproduction and amplification of the gene region of 514 base pairs were performed using FKSI-F1719 and FKSI-R2212 (16) and PCR primers by thermal cycler techne-TC-312 machine (Table 1). For verifying these functions, primers were controlled by conducting basic local alignment search tool BLAST in National Center for Biotechnology (NCBI) website.

Temperature cycles included the initial denaturation which was conducted under the temperature of 95°C for 30 seconds, followed by the below cycles in 35 rounds: denaturation (3 minutes under the temperature of 95°C), Connection (30 seconds under the temperature of 56/1°C), Reproduction (1 minute under the temperature of 72°C), and final reproduction (3 minutes under the temperature of 72°C).

Table 1. Primer sequences used in this work

Sequence	Oligonucleotide
CATTGCTGTGGCCACTTTAG	FKS1-F1719
GATTTCCATTCCGTGGTAGC	FKS1-R2212

Table 2. Distribution of absolute and relative abundance lesions taken from Candidiasis patients

Relative abundance	Absolute abundance	Clinical forms
32/7	17	Cutaneous
59/6	31	Mucosal
7/7	4	Visceral
100	52	Total

RESULTS

Identifying Strains as *Candida albicans* and Conducting the Test of Sensitivity via Broth Micro-Dilution Method

From among the total 52 samples taken from candidiasis patients, 37 samples (71%) belonged to women. Of all samples, mucosal lesions (59.6%) and visceral lesions (7.7%) were the most and least clinical forms of lesions respectively in this study (Table 2). With respect to the mucosal lesions, most samples were taken from vagina, and in visceral lesions, most samples were taken from phlegm. After studying samples, 41 samples (79% of all) were detected as *Candida albicans* and were studied in terms of their sensitivity to caspofungin using broth micro-dilution method. The MIC range detected for caspofungin, with 85% of *Candida albicans*, was MIC<0.5-2 in comparison with the range of reference strain(the MIC of the control strain was 0.25 µg/ml). Caspofungin with MIC of 0.5 micrograms per milliliter had the best inhibitory effect. Four samples in the yeast dilution of 10³cfu/ml showed the MIC of 4 and two cases showed the MIC of 8. In fact six samples (Ca3-Ca8-Ca10-Ca13-Ca16-Ca19) from among all 41 separated *Candida albicans* showed no sensitivity to caspofungin(Fig.1). Ca8 and Ca13 samples were taken respectively from CSF and phlegm of two men with prostate cancer. Ca3, Ca6, and Ca19 samples were taken from women with candidiasis vaginitis, and the Ca10 sample was also taken from lesions of a woman with lymphoma.

Separating Yeast DNA, PCR

After separating DNA, those *Candida albicans* resistant to caspofungin were separated with FKSI-F1719 and FKSI-R2212 primers. Then

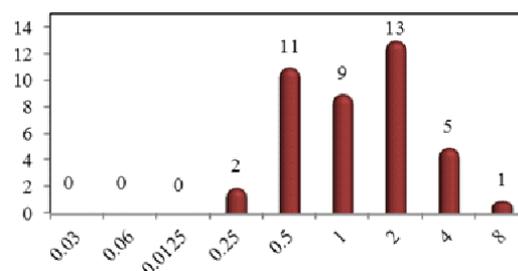


Fig.1. Susceptibility testing of 41 clinical isolates to caspofungin by CLSI and Microdilution Broth test.

a514-base pair was successfully enhanced using PCR for preparing the six separated *Candida albicans* DNAs.

DISCUSSION

Caspofungin is a semi-synthetic analogue from pneumocandin B0 with the high activity range against yeasts such as *Candida* and *Aspergillus* mold. In fact, the di-acetate salt comes from pneumocandin B0 with the molecule formula of $C_{52}H_{88}N_{10}O_{152}C_2H_4O_2$ and molecule weight of 42-1213 dalton. In most fungi, 1 β -D-glucan synthase is formed of two subunits: one is the catalytic subunit of connected to the plasma membrane (FKS), and one activator subunit which activates catalytic subunit by activating guanosine triphosphate. This process occurs with two glucan synthesis systems which are adjusted by FKS1 and FKS2 genes. The FKS1 gene appears during the normal growth of *Candida albicans*, and codes a large internal protein membrane which is subunit of GS complex (1 β -D-glucan synthase) and is inhibited by caspofungin. Therefore any change or mutation in this gene will lead to resistance of *albicans* species to it. Reduction of sensitivity to caspofungin is related to the sequence of amino acids in the FKS1p gene region, which is usually as a result of missenses mutation. These types of mutations in *Candida albicans* take place in two points of FKS protein and hot spots which are located in regions 640-650 and 1345-1365¹⁴⁻¹⁶.

The present study was conducted on 52 patients with suspected candidiasis who had mucosal, cutaneous, and visceral candidiasis and mostly had mucosal candidiasis. Tests proved that 41 cases were due to *Candida albicans*, out of which 3 cases were taken from cancer patients, 12 from women with vaginitis and the remainder from patients with other types of mucosal and visceral candidiasis. Of these patients, 23 had some history of reoccurrence of the infection despite receiving anti-fungal treatments, and most patients were women. The present study not only confirmed the increase of prevalence of *albicans* species in creating various types of candidiasis, but it also proved the increasing trend for resistance to anti-fungal medicine. Although caspofungin belongs to new anti-fungal antibiotics with an extensive application, resistance to it among fungi is

unfortunately increasing. The prevalence of acute and systemic candidiasis infections and consequently treatment with anti-fungal medicines and resistance to these combinations have more emphasized on the necessity to apply methods for determining medical sensitivity of fungal agents. Determining the sensitivity of the agent of the disease before starting the proper treatment and eradicating and eliminating fungal agents is a very profitable method which prevents indiscriminate use of medicines and consequently secondary and undesired medical resistance. In the present study, 15% of samples showed resistance to caspofungin while showing resistance to other anti-fungal medicines during treatment as well. According to studies, caspofungin has its best inhibiting effect for MIC of 0.5 micrograms per milliliter. One of the reasons for resistance of microorganisms such as fungi is mutation in their structural and regulatory genes.

Several resistance genes have so far been identified in *Candida albicans*, each leading to resistance of fungi to anti-fungal medicine with a separated molecule mechanism. Mutation in the FKS1 gene results to *Candida albicans* resistance to echinocandins such as caspofungin.

The resistance to caspofungin medicine in 41 *Candida albicans* strains taken from patients was found to be 15% through applying broth micro-dilution method. In fact, 6 resistant isolates were detected in these studies of which two strains of the samples were taken from visceral infections, three from mucosal infections, and one from cutaneous infections. Although being relatively small, the percentage of resistance is likely to be raised due to improper use of antibiotics or taking such medicine to prevent diseases especially in special patients.

Applying molecule methods and studying the pattern of gene expression has nowadays been a great aid in timely detection of infections such as fungal infections.

In a study by Sergey, V, 85 *Candida albicans* isolates resistant to caspofungin were studied and the reason for their resistance was reported to be mutation in their FKS1 gene, as in 93% of them, the mutation had occurred in ser645 region in this gene, while 6% and 1% of the mutations had occurred in the regions of 641 and 644 of the said gene respectively¹⁶. In a separate

study conducted by Marie Desnos *et al.*, over 25 *Candida albicans* taken from different surface and sub-surface samples of patients, the missense mutation was observed in 8 isolates³⁰.

In two other studies conducted simultaneously in 2006, Laverdier and Balashov reported most mutations in *Candida albicans* genome to have occurred in the regions of 1361 arginine and 644 lucine³¹.

Taking the results of the present study, it could be generally concluded that a combination of broth micro-dilution method and the molecule method for polymerases chain reaction not only is a reliable method rapid and correct detection of isolates resistant to anti-fungal agents, but it also makes it possible to investigate the mechanisms of medical resistance and the genes involved in them.

Hence, it is suggested that researchers continue to carry out these types of research for boosting the quality of fungal infection treatments and also for making the epidemiologic information of these diseases more desirable to witness significant success in treatments.

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