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Original Article

Antifungal Susceptibility Analysis of Clinical Isolates of *Candida parapsilosis* in Iran

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Abstract

Background: *Candida parapsilosis* is an emergent agent of invasive fungal infections. This yeast is one of the five most widespread yeasts concerned in invasive candidiasis.

C. parapsilosis stands out as the second most common yeast species isolated from patients with bloodstream infections especially in neonates with catheter.

Recently several reports suggested that its reduced susceptibility to azoles and polyene might become a cause for clinical concern, although *C. parapsilosis* is not believed to be intensely prone to the development of antifungal resistance.

Methods: In the present report, One hundred and twenty clinical isolates of *C. parapsilosis* complex were identified and differentiated by using PCR-RFLP analysis. The isolates were then analyzed to determine their susceptibility profile to fluconazole (FLU), itraconazole (ITC) and amphotericin B. The minimum inhibitory concentration (MIC) results were analyzed according to the standard CLSI guide.

Results: All of isolates were identified as *C. parapsilosis*. No *C. metapsilosis* and *C. orthopsilosis* strains were found. Evaluation of the antifungal susceptibility profile showed that only three (2.5%) *C. parapsilosis* were resistant to fluconazole, three (2.5%) *C. parapsilosis* were resistant to itraconazole and two (1.7%) *C. parapsilosis* were amphotericin B resistant.

Conclusion: Profiles in clinical isolates of *C. parapsilosis* can provide important information for the control of antifungal resistance as well as distribution and susceptibility profiles in populations.

Keywords: *Candida parapsilosis*, Antifungal susceptibility, Resistant, Iran

Introduction

Candidiasis is caused by *Candida* species that can produce a broad spectrum of human infections (1). *Candida parapsilosis* is among the most common yeasts concerning invasive candidiasis. *C. parapsilosis* is the most common non-*albicans Candida* species, frequently isolated from individuals with damaged immune systems and from pediatric units. Recently, the incidence of invasive candidiasis due to *C. parapsilosis* has been increased (2). The high affinity of *C. parapsilosis* for intravascular devices

may lead to exogenous candidemia due to contamination of such devices or because of biofilm (3-9).

The pathogen is also able to form biofilms on other medical surface such as implants. Based on new taxonomic concepts, *C. parapsilosis* is a complex consists of *C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis* in which *C. parapsilosis* are mentioned as the predominant species isolated from clinical samples (10, 11).

Azole antifungals including fluconazole, itraconazole, and miconazole and polyene antibiotics such as amphotericin B and nystatin are commonly drugs used for treatment of candidiasis in forms of topical, oral or intravenous. Fluconazole has been widely used for the treatment of systemic candidiasis (12). These medicines are indicated to inhibit the activity of lanosterol demethylase enzyme, which involved in the biosynthesis of ergosterol in fungal cells (13). However, several recent studies have revealed an increasing resistance to this antifungal.

Although in comparison to other *Candida* species, there is little information on antifungal resistance in *C. parapsilosis*, evidences have indicated that this pathogenic yeast is prone for development of drug resistance and the frequency of such resistances may differ by geographic region (14).

Antifungal agents prevent from infections caused by *Candida* in high-risk patients but the increasing use of antifungals might also have changes in the species causing infections. The hands of healthcare workers may be the main environmental source that in nosocomial outbreaks of *C. parapsilosis* have also been described formerly (10).

Few studies have informed about antifungal susceptibility profile of *C. parapsilosis* in Iran. Moreover, the definition of the antifungal susceptibility profile of *C. parapsilosis* is significant for clinical. The differences in susceptibility this species between antifungal agents may influence the therapeutic choices. Because of this change and the importance of *C. parapsilosis* in Iran, we surveyed the susceptibilities of such isolates to three commonly used antifungal agents, namely, FLU, ITC and AMB.

Materials and Methods

Clinical isolates

A total of 120 isolates of *C. parapsilosis* already collected and diagnosed from clinical samples during an epidemiological study in Tehran, Isfahan, Mazandaran, and Alborz provinces between 2009 to 2013 (1), were used in this study.

All isolates were sub-cultured on CHROMagar *Candida* medium (CHROMagar Microbiology, Paris, France), incubated at 35 °C for 48 h and production of specific colony colors were analyzed for preliminary species identification.

DNA extraction

The isolated yeasts were sub-cultured on sabouraud dextrose agar (Merck, Germany) at 32 °C for 48 h. Genomic DNA was extracted from harvested yeasts grown in a broth comprised of 2% glucose, 1% yeast extract (Difco, Detroit, Mich.) and 2% pepton (Oxoid). Briefly, yeast cells were harvested and lysed adding 0.3 g of glass beads (diameter, 0.45 to 0.52 mm; Sigma, St. Louis, MO), 300 µl of DNA lysis buffer (100mM Tris-HCl, pH 8.0, 2% Triton X-100, 1% sodium dodecyl sulfate, 1mM EDTA), and 300 µl of phenol-chloroform-isoamyl alcohol (PCI) (25:24:1). The mixture was then vortexed for 30 sec and centrifuged at 5,000 rpm for 5 min. The supernatant was collected, 300 µl of chloroform was added, and vortexed for few seconds and centrifuged again; then 250 µl of ethanol and 25 µl of 3M sodium acetate (pH 5.2) were added to the obtained supernatant and incubated for 10 min at -20°C. The mixture was then centrifuged at 12,000 rpm for 12 min and the pellet was re-suspended in 100 µl distilled water as purified DNA and stored at -20 °C until used (15).

Species confirmation

In order to confirmation of the already identified *C. parapsilosis* isolates and for molecular differentiation of *C. parapsilosis* from *C. orthopsilosis*, and *C. metapsilosis*, PCR-RFLP was performed as described previously (1, 11, 16, 2). Briefly, PCR mixture containing 5 µl of 10× reaction buffer, 1mM dNTPs, 1mM MgCl, 1U of DNA *Taq* polymerase, 2µl of each ITS1 (5 ' -TCCGTAGGTGAACCTGCGG-

3 '), ITS4 (5 ' -TCCTCCGCTTATTGATATGC-3 ') primers, or 2µl of each SADHF (5 ' -GTT GAT GCT GTT GGA TTGT-3 '), SADHR (5 ' -CAA TGC CAA ATC TCC CAA-3 ') primers (10, 15), and 3 µl of extracted DNA in a final volume of 50 µl, were used.

Conditions for PCR in thermal cycler machine were as follows: initial DNA denaturation at 95° C for 4 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s and extension at 72 °C for 1 min and final extension step at 72°C for 10 min.

RFLP mixture contained 10µl of PCR product, 2µl of the supplied buffer, 1U of *Nla*III and/or *Msp*I enzymes, and 18µl of nuclease-free distill water.

Five µl of PCR product and 15 µl of each RFLP products were fractionated by electrophoresis on respectively 1% and 2% agarose gel and stained with 0.5µg/ml ethidium bromide. The species of *C. parapsilosis* was confirmed or identified according to already published electrophoresis patterns (1, 2).

Antifungals

Standard fluconazole (FLC) (Tehran Daru, IRAN), amphotericin B (AMB) (Sigma-Aldrich, USA), itraconazole (ITC) (Tehran Daru, IRAN) were used for preparation of tock solutions. FLC was prepared in distilled water while AMB and ITC in dimethyl sulfoxide (DMSO). The solutions were then kept frozen at -70 °C until use. Dilution of antifungal was performed with RPMI 1640 medium (Invitrogen, GIBCO) and buffered to pH 7.0 with 0.165 M morpholine propane sulfonic acid (MOPS) buffer (Sigma, USA) (10).

Antifungal susceptibility analysis

Antifungal susceptibility tests were performed by broth micro dilution method as described in Clinical and Laboratory Standards Institute (CLSI) guidelines, document M27-S3 (17).

C. parapsilosis ATCC 22019-type strain was used for quality control in all antifungal susceptibility tests.

Tests were performed in 96-well round-bottom microtiter plates.

Cell suspensions were prepared in RPMI 1640 medium and were adjusted to give a final inoculum concentration of about 0.5×10^3 to 2.5×10^3 cells/ml. The plates were incubated at 35 °C and were observed after 48 h. minimum inhibitory concentration (MIC) were then determined and compared with drug-free control. All tests were performed in duplicate. The MIC results were analyzed according to the M27-S3 supplement of the CLSI Guide.

The interpretive criteria for susceptibility to antifungal drugs were based on CLSI (17).

Results

One hundred and twenty clinical isolates of *C. parapsilosis* were studied. These isolates which had been collected during epidemiological studies and diagnosed as *C. parapsilosis* previously, were obtained from 120 patients (65% females and 35% males). Based on the age, the patients ranged from 6 to 81 yr with a mean age of 42.4 yr. The location and gender of these clinical isolates are mentioned in Table 1.

Table 1: *C. Parapsilosis* isolates according to gender and location

Location	Gender				Total	
	Female		Male			
	n	%	n	%	n	%
Nail	66	55	22	18.3	88	73.33
Groin	2	1.7	6	5	8	6.7
Interdigital	2	1.7	9	7.5	11	9.2
Vaginitis	3	2.5	0	0	3	2.5
Hand	2	1.7	2	1.6	4	3.3
Others (sputum,ear discharge)	3	2.5	3	2.5	6	5
Total	78	65.1	42	34.9	120	100

RFLP analysis

PCR using the primers ITS1 and 4 followed by restriction digestion with *MspI* indicated a restriction map which meets the restriction characteristics of *C. parapsilosis sensu stricto* (data not shown). In addition, amplification of *SADH* gene revealed a single band with approximate size of 716bp in all isolates (Fig 1.A). Amplification and subsequent digestion of the *SADH* gene with *Nla*III *(Hin1*II) in 120 strains *C. parapsilosis* complex produced fragments with the sizes 131 and 505bp. No *C. metapsilosis* and *C. orthopsilosis* strains were found (Fig.1.B).

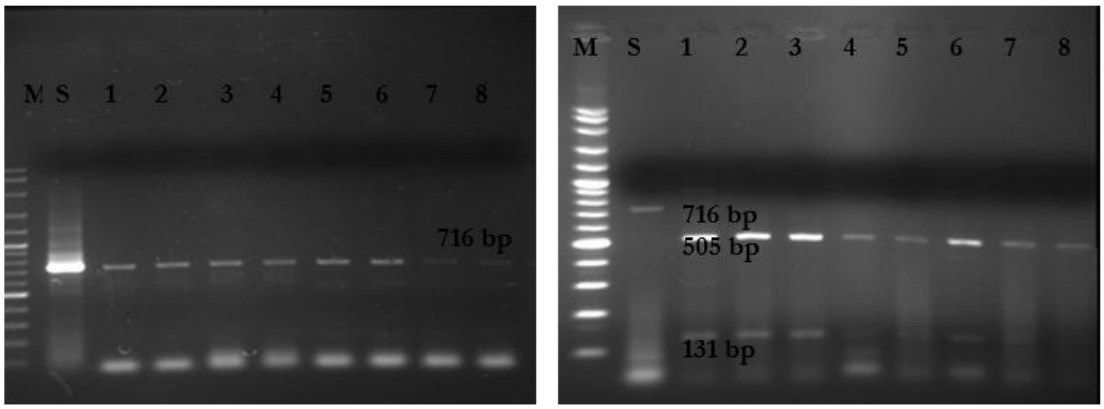

Fig. 1: a) Agarose gel electrophoresis of *SADH*-PCR products of *C. parapsilosis* complex. Lane M is 100 bp DNA size marker, Lane1(S) is *C. parapsilosis* ATCC 22019, lanes 2– 8 are *C. parapsilosis* clinical isolates.b) RFLP-PCR products of *C. Parapsilosis* after digestion with *Nla*III. Lane M is 100 bp DNA size marker, lane 1 is *C. Parapsilosis* ATCC 22019 without effect enzyme (716 bp), Lanes 2-8 are *C. Parapsilosis* (131 and 505 bp).

Antifungal susceptibility

The results of antifungal susceptibility analysis are described in Table 2. Two, three and three isolates were found as the strains resistant AMB, FLC, and ITC, respectively.

Table 2 summarizes the in vitro susceptibilities of the 120 isolates to the three antifungal drugs as measured by the reference broth micro dilution method. The data are reported as MIC ranges of the isolates are inhibited.

Table 2: The results of antifungal susceptibility of clinical isolates of *C. parapsilosis* used in this study

Antifungal Agent	Susceptible* MIC (mg/ml)	Susceptible Dose dependent* MIC (mg/ml)	Resistant* MIC (mg/ml)	Number of tested isolates	Number of resistant isolates
Amphotericin B	≤0.125	0.25-0.5	≥1	120	2 (≥1)
Fluconazole	≤8	16-32	≥64	120	3 (>64)
Itraconazole	≤0.125	0.25-0.5	≥1	120	3 (>1)

Of the 120 isolates tested, for two isolates the broth microdilution MICs were ≥1mg/ml for amphotericin B and for 3 isolates the fluconazole MICs were ≥64 mg/ml, for 3 isolates the itraconazole MICs were ≥1mg/ml. Numbers of susceptible, susceptible in a dose-dependent manner and resistant isolates are shown in this chart. Surprisingly, all eight resistant species were isolated from the patients with nails infection.

Discussion

C. parapsilosis is the second common species isolated from patients with candidemia (4, 18, 19). The prevalence of fungemia due to this fungus has changed over the years and has been reported frequently in blood stream infections especially in neonates with catheter. The reasons for the rising incidence of *C. parapsilosis* candidemia are not completely known, although catheters and parenteral nutrition have been known as specific risk factors (4-7, 9, 18, 20-22).

The source of most occurrences of *C. parapsilosis* fungemia is a vascular catheter and these commonly progress in patients who had formerly received antifungal treatment. Therefore, the administration of the antifungal agents should be combined with the quick removal of the catheter to obtain an acceptable response to FLU or to any other antifungal (10).

C. parapsilosis can be part of the normal flora of the human skin, appearing to be directly presented into the bloodstream and is often associated to an exogenous source (hands of healthcare providers). BSIs caused by *C. parapsilosis* are not commonly related with previous colonization. However, infections caused by *C. albicans* frequently result from endogenous sources (commonly colonizing the genital mucosae and gastrointestinal) (5, 6, 9).

In comparison with risk factors related of *C. albicans*, these in *C. parapsilosis* fungemia were transplant recipients, patients who got previous antifungal therapy and especially in neonates who received parenteral nutrition. Rate of mortality reported with *C. parapsilosis* fungemia is very low (4).

We evaluated the in vitro activities of FLC, ITC, and AMB against *C. parapsilosis*. According to the results of Mirhendi et al (2), we analyzed the restriction polymorphism of the *SADH* gene and successfully distinguished *C. parapsilosis* from *C. metapsilosis* and *C. orthopsilosis*. Previous studies in Iran had already described normal or high sensitivity of Iranian isolates of *C. parapsilosis* to FLC, ITC and AMB (10, 23-25).

Azole and polyen resistance were detected among all *C. parapsilosis* isolates. Three isolates were resistant to FLC (MIC ≥64 μg/ml), and three isolates were resistant to ITC (MIC ≥1 μg/ml). Only two *C. parapsilosis* were resistant to AMB. Monitoring of the susceptibility profiles of *C. parapsilosis* must highly advisable, although in this study FLU and ITR showed high sensitivity against *C. parapsilosis*. The combination of the ability to develop biofilms on catheters and other medical indwelling devices and decreased susceptibility to azole may become problematic (10).

Decreased susceptibilities to fluconazole, itraconazole, and amphotericin B were shown among *C. parapsilosis* isolates in all age groups in this present study. Continuing surveillance is critical to limit resistance among isolates (26). These findings confirm the worldwide reports of low levels of FLC resistance in *C. parapsilosis* isolated from blood cultures (20, 27, 28). In this study, the majority of isolates were fluconazole susceptible; Thus FLC is a reasonable drug for treatment of *C. parapsilosis* infections (4). However, other investigations indicated susceptible-dose dependent or resistance of *C. parapsilosis* isolates to FLC and AMB (10).

Pfaller et al. found no evidence of increasing azole resistance over time among *C. parapsilosis* isolates in their investigation in 124 medical centers worldwide (3). Another study revealed the susceptibility of *C. parapsilosis* isolates from fungemia in a Spanish hospital 100% for AMB, 95.6% for FLC, and 76.5% for ITC (29). Other investigations reported almost similar results (3, 4, 30).

Profiles in clinical isolates of *C. parapsilosis* may provide significant information for the control of antifungal resistance as well as distribution and susceptibility profiles in populations (8, 12). In addition, understanding of the drug susceptibility profiles particularly in azole and polyen resistances, help us to improve prophylactic and antifungal therapeutic strategies (7, 31).

Conclusion

This study presented antifungal susceptibility of clinical isolates of Candida parapsilosis in Iran. Decreased susceptibilities to fluconazole, itraconazole, and amphotericin B were shown in this present study. The majority of isolates were fluconazole

susceptible. Thus, fluconazole is a practical drug for treatment of *C. parapsilosis* infections.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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