Medical Mycology August 2013, **51**, 657–663

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Molecular identification and distribution profile of *Candida* species isolated from Iranian patients

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A total of 855 yeast strains isolated from different clinical specimens, mainly nail (42%) and vulva-vagina (25%) were identified by a set of polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP). Genomic DNA was extracted from fresh colonies using Whatman FTA Card technology. PCR assays were performed on the complete ribosomal DNA internal transcribed spacer (rDNA-ITS) region for all isolates and species identification was carried out through their specific electrophoretic profiles after digestion with the enzyme *Msp*I. Those isolates suspected as *Candida parapsilosis* group were then subjected to amplification of the secondary alcohol dehydrogenase (*SADH*) gene and restriction digestion with *Nla*III enzyme. In total, 71.1% of the strains were obtained from females and 28.9% from males. The age group of 31–40 years consisted of the highest frequency of patients with candidiasis. *Candida albicans* was the predominant species (58.6%) followed by *C. parapsilosis* (11.0%), *C. glabrata* (8.3%), *C. tropicalis* (7.0%), *C. kefyr* (5.8%), *C. krusei* (4.4%), *C. orthopsilosis* (2.1%), and *C. guilliermondii* (0.6%). A few strains of *C. lusitaniae*, *C. rugosa*, *C. intermedia*, *C. inconspicua*, *C. neoformans* and *S. cerevisiae* were isolated. We could not identify 8 (0.9%) isolates. *Candida albicans* remains the most frequently species isolated from Iranian patients; however, the number of non-*C. albicans Candida* species looks to be increasing. The simple and reliable PCR-RFLP system used in the study has the potential to identify most clinically isolated yeasts.

Keywords Microbial epidemiology, *Candida* species, Iran

Introduction

Candida species are ubiquitous yeasts that can cause a broad spectrum of human infections, known as candidiasis. This genus includes at least 30 species of medical importance that have been implicated in human candidiasis [1,2]. During the past several decades, the incidence of infections caused by the genus *Candida* has substantially increased due to expanding immunosuppressive situations, and those species once thought to be non-pathogenic are currently considered opportunistic pathogens [2]. Considering variations in susceptibilities to antifungal agents among *Candida* species, identification to species level is important to ensure quick and appropriate treatment [3]. There are several epidemiological studies focused on the isolation of *Candida* from certain clinical conditions or specimens [4,5], while the epidemiologic data pertaining to distribution of *Candida* species at different body sites are limited [3].

Since studies regarding epidemiology of infectious *Candida* species were not well documented in Iran, in this report we present the species distribution of clinical strains

Received 18 May 2012; Received in revised final form 23 November 2012; Accepted 10 January 2012

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DOI: 10.3109/13693786.2013.770603

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of *Candida*, obtained from four different Iranian provinces. We used a set of polymerase chain reaction (PCR) amplifications of the entire internal transcribed spacer (ITS)-rDNA region and secondary alcohol dehydrogenase (*SADH*) gene, followed by restriction fragment length polymorphism (RFLP) for species delineation of the isolates.

Materials and methods

A total of 855 yeast strains isolated between June 2009 to June 2010 from patients with various clinical forms of candidiasis in Tehran, Alborz, Isfahan, and Mazandaran provinces were included in the study. The samples were collected from the accessible specimens, mainly from mucocutaneous infections. The isolates were randomly collected in different periods of time; therefore, they are not the exact representative of distribution of clinical forms of candidiasis. In addition, 18 reference strains of medically important yeasts were used as controls (Table 1), all supplied by Teikyo University Institute of Medical Mycology (TIMM), Tokyo, Japan. DNASIS software (Hitachi Software Engineering Co., Ltd, Tokyo, Japan) was used for analyzing several GenBank sequences of different yeasts (Table 1) to determine the size of entire ITS1-5.8SrDNA-ITS2 region before and after *in silico* digestion with the restriction enzyme *Msp*I.

The yeasts were subcultured on Sabouraud glucose agar (Difco, Detroit, MI, USA) and incubated at 30°C for 48 h. Genomic DNA was isolated using FTA® Elute MicroCards (Whatman Inc., Clifton, NJ, USA) [6] in accordance with the manufacturer's instructions with minor modifications. Briefly, a loopful of fresh colony was suspended in 100 μl of sterile water and 4 μl of the suspension was dropped on a disc of FTA card (3 mm in diameter) and incubated at room temperature for at least 3 h. The dried papers were eluted in 500 μl distilled water for a few seconds, then the paper was transferred to a new tube containing 30 μl distilled water and incubated at 94°C for 20 min. The paper discs were removed and the water containing DNA was used for PCR or stored at −20°C until used.

For molecular identification of common *Candida* species, the already described PCR-RFLP profiles were used [7,8]. Briefly, the contiguous ITS1-5.8SrDNA-ITS2 region was amplified using PCR mixture containing 5 μl of 10× reaction buffer, 1.5 mM $MgCl_2$, 0.4 mM dNTPs, 2.5 U of DNA *Taq* polymerase, 30 pmol of each ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) primers [9] and 3 μl of extracted DNA in a final volume of 50 μl. Conditions for PCR were as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s, and extension at 72°C for 1 min, with a final extension step at 72°C for 7 min. An aliquot of 10 μl of each ITS-amplicon was digested with the restriction enzyme *Msp*I (Fermentas, Vilnius, Lithuania). For definite identification of newly described species in the *C. parapsilosis* complex, the *SADH* gene was amplified using the SADHF (5′-GTT GAT GCT GTT GGA TTG T-3′) and SADHR (5′-CAA TGC CAA ATC TCC CAA-3′) primers [10], followed by digestion with *Nla*III restriction enzyme [8]. Aliquots of 5 μl of PCR amplicons and 12 μl of each RFLP products were fractionated by electrophoresis on 1.5 and 2% agarose gel, respectively, and stained with 0.5 μg/ml ethidium bromide.

To confirm the reliability of molecular methods used for identification of common *Candida* species, a total of 100 isolates including *C. albicans* ($n = 40$) and *C. tropicalis*, *C. krusei* and *C. glabrata* ($n = 20$ for each species) were subcultured on CHROMagar Candida (CHROMagar Microbiology, Paris, France), incubated at 35°C for 2 d and observed for specific colony colors.

Results and discussion

Table 1 shows the size of ITS region for common and rare pathogenic *Candida* and a number of other pathogenic yeasts before and after computer endonuclease digestion with *Msp*I. Figure 1 demonstrates the patterns of ITS-PCR-RFLP for reference yeast strains after digestion with *Msp*I. As shown, the fragment lengths were exactly the same as the estimated sizes in the computational sequence analysis (Table 1). Then, considering the size of fragments obtained from restriction digestion of ITS-PCR products by an inexpensive enzyme (*Msp*I), a wide range of common and some rare pathogenic yeasts including the major *Candida* species were differentiated. The limitation of the method is similar fragment size in some uncommon species of *Candida*. For example, the size of ITS before and after digestion is similar in *C. intermedia*, *C. rugosa* and *C. lusitaniae*. Also, some uncommon yeasts like *C. kefyr* and *C. famata* had no restriction enzyme cutting site for *Msp*I; nevertheless, the size of ITS was helpful to presume the species (Table 1 and Fig. 1).

The result of species identification observed on CHROMagar Candida for selected *Candida* isolates was 100% concordant with the species delineation in molecular methods used (data not shown).

Of the 855 different clinical isolates, 606 (71.1%) were from females. The majority of strains were recovered from nail and other mucocutaneous samples (Table 2). Patients in the age groups of 31–40 and 81–90 years had the highest and lowest frequency of positive samples, respectively. Amplification and subsequent digestion of the ITS region with *Msp*I generated two-band patterns, characteristic for *C. albicans* ($n = 501$), *C. glabrata* ($n = 71$), *C. tropicalis* ($n = 60$), *C. krusei* ($n = 38$), and a three-band pattern

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Table 1 Yeast species and related ITS sequences used in this study for *in silico* and laboratory *Msp*I-RFLP analysis.

Yeast species	Size of ITS	Size of *Msp*I-RFLP fragments	Reference strains used in this study	Examples of GenBank accession numbers used in the sequence analysis
Candida albicans	537	239, 298	TIMM 1768	AB369915, GQ280312, HE860439, JN606261, JN606273, HE860439, JN606276, JN606275, JN606295, JN606265, JN606268, JN606263, JN606256, JN606257
Candida tropicalis	526	186, 340	ATCC 750	HM231275, GQ376071, EU924133, AY939810, HQ014734
Candida glabrata	881	320, 561	ATCC 90030	AY939793, FN652301, GQ376080, AM492798, AM492797, AY198398
Candida nivariensis	760	205, 236, 319	CBS 10161	FM955316, GU199441, GU199442, GU199443, GU199444
Candida bracarensis	805	253, 552	CBS 10154	GU199439, GU199440, GU199438, JN882340, AY589573
Candida krusei	510	250, 260	ATCC 6258	AB369918, AY939808, JX174414, FM199964, FM199965, EF136369, FJ515204, L47113
Candida parapsilosis	530	530	ATCC 22019	EU564209, AB109277, DQ681369, DQ681358, EU871507, AM117819, AB109267
Candida orthopsilosis	510	510	–	FM178398, FM178395, FM178394, GQ152298, GQ152293, GQ152292, GQ152291, FJ872018, FJ872017, FJ515170, FM172983, EU564208, FM178400
Candida metapsilosis	531	531	–	AY391849, JQ585714, GQ152299, GQ152297, FJ872019, FJ515169, EU564207, EU484055, FJ515200
Candida guilliermondii	607	82, 155, 370	TIMM 3400	JN183444, EU568971, EF191048, DQ663478, DQ680842, AY939795, AY939792, GQ334393, FJ969194, EU568973, EU177579
Candida lusitaniae	382	118, 264	TIMM 3479	JF508436, GQ376072, EU568925, DQ223426, HQ693786, HQ693785, EF221824
Candida famata	639	639	JCM 1439	HE681099, FR686594, EU149789, JQ912667, HE681104, HE681103, GQ458025
Candida kefyr	720	720	TIMM 0300	AF543841, HE650694, HE650693, HE650691, HQ396523, AY939806, JQ425346, HQ014731, EU019227
Cryptococcus neoformans	555	127, 428	ATCC 90113	JN939455, FR751339, AF356652, AJ876525, AJ876598, AY973273
Candida norvegensis	493	8, 227,258	JCM 2309	AB179768, AB278162, AB278164, AB278163
Candida inconspicua	455	8, 208, 239	JCM 9555	AB179767, AJ853766
Candida intermedia	389	122, 267	JCM 1607	AY500373, EF568011, JQ726602, EF192222
Saccharomyces cerevisiae	840	124, 716	ATCC 9763	FN393995, GQ376091, FJ793809, EU649673, AM262829, AM262828, AM262827, AY525600
Candida rugosa	399	121, 278	JCM 1619	GU144663, FJ768914, AB727605, AY500374
Candida viswanathii	484	25, 153, 306	JCM 9567	DQ24920

TIMM, Teikyo University Institute of Medical Mycology; ATCC, American Type Culture Collection; CBS, Centraalbureau voor Schimmelcultures; JCM, Japan Collection of Microorganisms.

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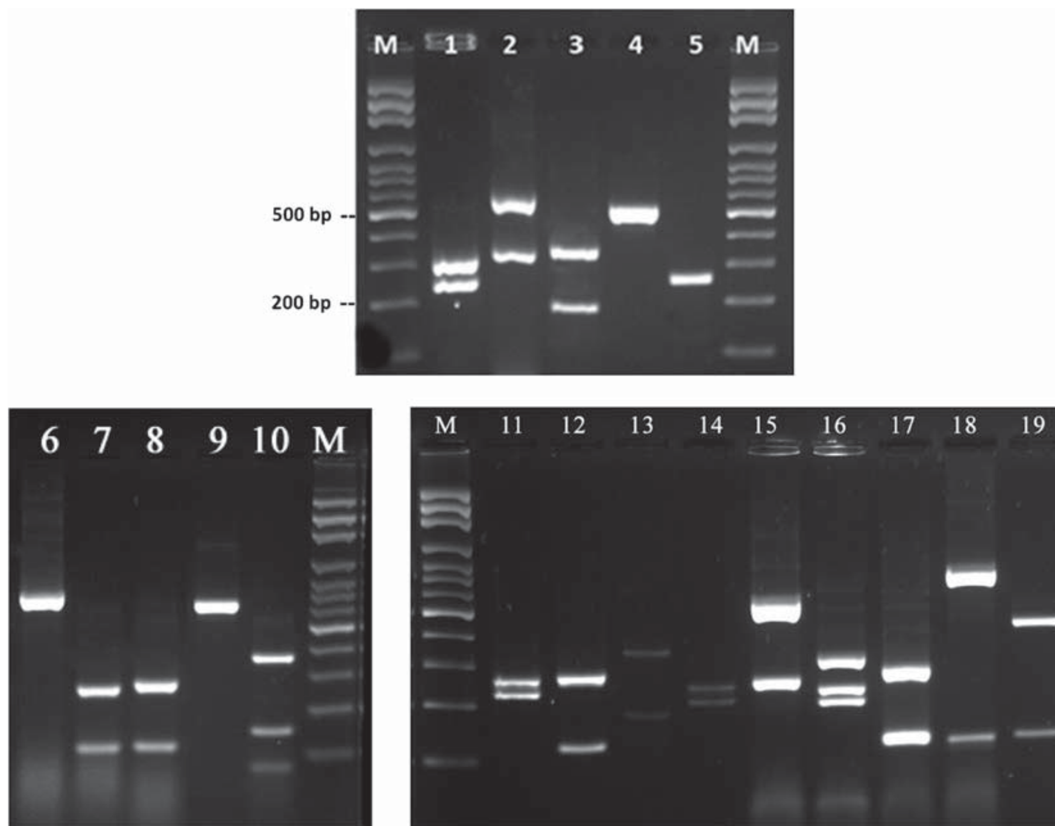

Fig. 1 Agarose gel electrophoresis of ITS-PCR products of various pathogenic yeast species after digestion with *Msp*I. Lanes 1–19 are *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. kefyr*, *C. lusitaniae*, *C. rugosa*, *C. famata*, *C. guilliermondii*, *C. norvegensis*, *C. intermedia*, *C. viswanathii*, *C. inconspicua*, *C. bracarensis*, *C. nivariensis*, *C. lusitaniae*, *Saccharomyces cerevisiae* and *Cryptococcus neoformans*, respectively. Lanes M are 100 bp DNA size marker.

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specific for *C. guilliermondii* ($n = 5$) but a single band (no cutting site) was observed for *C. parapsilosis* complex ($n = 112$), *C. kefyr* ($n = 50$), and *C. famata* ($n = 1$) . Strains of *C. kefyr* created an approximately 720 bp band and were differentiated from the *C. parapsilosis* complex, which produced a 520 bp band [7]. Thus, using a combination of ITS-RFLP with *Msp*I and ITS-amplicon size, the majority of the strains were identified as six distinct species. Amplification and subsequent digestion of the *SADH* gene with *Nla*III in 112 remaining strains, suspected to be *C. parapsilosis* complex, produced fragments with two different patterns characteristic for *C. parapsilosis* ($n = 94$) and *C. orthopsilosis* ($n = 18$). No *C. metapsilosis* strains were found. A few strains of *C. rugosa* ($n = 3$), *C. lusitaniae/C. intermedia* ($n = 2$), *C. inconspicua* ($n = 1$), *Cryptococcus neoformans* ($n = 1$), and *Saccharomyces cerevisiae* ($n = 2$) were isolated. We could not identify a total of eight (0.9%) isolates because of undefined RFLP patterns (data not shown).

Candida albicans has historically counted for 70–80% of strains recovered from candidiasis; nonetheless, over the last decades non-*C. albicans Candida* species (NACs) have frequently emerged as human pathogens [1]. In the present study, *C. albicans* was the prominent isolate (58.8%) in all clinical samples and NACs included *C. parapsilosis*, *C. glabrata*, *C. kefyr*, *C. tropicalis*, *C. krusei*, *C. orthopsilosis* and *C. guilliermondii*. The isolates were frequently recovered from non-sterile body samples. Most of the *C. albicans* strains (66%) were isolated from *Candida* vulvovaginitis (CVV) and onychomycosis. In general, more than 70% of CVV cases are caused by *C. albicans* worldwide, while NACs like *C. glabrata* are less frequently responsible for fungal vaginitis [11]. Likewise, *C. albicans* (82%) and *C. glabrata* (13.5%) were the major causative agents of CVV in our study. *Candida albicans* also comprises more than 50% of all urinary tract isolates [11] while congruent with the study of Ozhak-Baysan *et al.* [12], 72% of encountered candiduria cases in our investigation were caused by NACs. Similar to a study performed in Mexico [13], the incidence of onychomycosis by NACs in our analysis (56%) was dominated by *C. albicans*. All these data accentuate the increasing

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Table 2 Distribution profile of yeast species isolated in this study by clinical sample.

Isolated species	*C. albicans*	*C. glabrata*	*C. tropicalis*	*C. krusei*	*C. parapsilosis*	*C. orthopsilosis*	*C. kefyr*	*C. guilliermondii*	*C. intermedia/ lusitaniae*	*C. famata*	*C. rugosa*	*C. inconspicua*	*C. neoformans*	*S. cerevisiae*	*unknown*	Total (%)
Clinical sample (Body site)																
Ungual candidiasis																
Nail	154	10	46	25	67	15	26	5	2	–	3	–	–	1	5	359 (42.0)
Mucosal candidiasis																
Vulva-vagina	177	29	–	2	1	–	5	–	–	–	–	–	–	–	–	214 (25.0)
Mouth	12	1	–	–	–	–	3	–	–	–	–	–	–	–	–	16 (1.9)
Nose mucus	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1 (0.1)
Esophagus	3	–	–	–	1	–	–	–	–	–	–	–	–	–	–	4 (0.5)
Sputum	30	12	1	1	1	–	3	–	–	–	–	–	–	–	2	50 (5.8)
BAL	24	6	–	–	–	–	2	–	–	–	–	1	–	1	–	34 (4.0)
Sinus	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	1 (0.1)
Cutaneous candidiasis																
Scalp	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2 (0.2)
Hand	6	–	1	1	3	–	2	–	–	–	–	–	–	–	–	13 (1.5)
External ear discharge	–	–	–	–	3	–	–	–	–	–	–	–	–	–	–	3 (0.4)
Sole	3	–	–	–	–	–	2	–	–	–	–	–	–	–	–	5 (0.6)
Calf	1	–	–	–	2	–	–	–	–	–	–	–	–	–	–	3 (0.4)
Groin	34	3	–	–	5	1	1	–	–	–	–	–	–	–	1	45 (5.3)
Mammary folds	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2 (0.2)
Genital folds	3	1	–	–	–	–	–	–	–	–	–	–	–	–	–	4 (0.5)
Sore	5	1	1	–	–	–	–	–	–	1	–	–	–	–	–	8 (1.0)
Interdigital spaces	13	–	–	1	6	1	1	–	–	–	–	–	–	–	–	22 (2.6)
Systemic candidiasis																
Urine	8	4	7	7	–	–	3	–	–	–	–	–	–	–	–	29 (3.4)
Blood	14	3	4	–	3	–	2	–	–	–	–	–	–	–	–	26 (3.0)
Cornea	4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	4 (0.5)
Dialysis fluid	–	–	–	–	2	–	–	–	–	–	–	–	–	–	–	2 (0.2)
Peritoneal fluid	2	1	–	–	–	1	–	–	–	–	–	–	–	–	–	4 (0.5)
Vitreous fluid	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1 (0.1)
Eye discharge	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1 (0.1)
Biopsy of lung transplantation	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1 (0.1)
Cerebrospinal fluids (CSF)	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–	1 (0.1)
Total (%)	501 (58.6)	71 (8.3)	60 (7.0)	38 (4.4)	94 (11.0)	18 (2.1)	50 (5.8)	5 (0.6)	2 (0.2)	1 (0.1)	3 (0.4)	1 (0.1)	1 (0.1)	2 (0.2)	8 (0.9)	855 (100)

trend toward the isolation of NACs along with decrease of *C. albicans* in urine and nail infections.

Candida parapsilosis complex were the second most common isolates and the most abundant of NACs. The complex was reclassified into three genetically segregated species; *C. parapsilosis sensu stricto*, *C. metapsilosis* and *C. orthopsilosis* [10]. Accordingly, data regarding the distribution of these species are limited so far. In accordance with many previous reports [14,15], *C. parapsilosis sensu stricto* was the dominant species of the complex in the present survey followed by *C. orthopsilosis*. The species was considered as the first or second most frequent agent of *Candida* onychomycosis in some South American countries [16] and of the most commonly recovered yeasts from hands. In the confirmation of these reports, *C. parapsilosis* was the second most abundant pathogen of *Candida* onychomycosis and hand infection, outranked only by *C. albicans* in this study. Similar to the study of Feng *et al*. [17], *C. orthopsilosis* was only isolated from cutaneous samples (skin and nail) of dermatological outpatients, suggesting a possible pathogenic role of the species in superficial infections. It is noteworthy that none of the *C. parapsilosis* complex isolates were *C. metapsilosis*. Based on recent data, *C. metapsilosis* is an environmental and less virulent organism, whereas *C. parapsilosis* and *C. orthopsilosis* have adapted to mammalian niches [16]. However, contrary to our finding, some studies found high prevalence of *C. metapsilosis*, accompanied with low frequency of *C. orthopsilosis* [18]. This may be correlated with species distribution of the *C. parapsilosis* complex varying geographically.

Prevalence of mucosal infections caused by *C. glabrata* has notably risen [1] and this fact was well reflected in our findings where 67% of the strains ($n = 48$) were isolated from mucosal candidiasis. Genetic studies resulted in the discovery of the fact that *C. glabrata* is a complex of species including two 'new cryptic species': *C. bracarensis* [19] and *C. nivariensis* [20]. However, in regard to the size difference of ITS-amplicons [21] and ITS-RFLP patterns by *Msp*I (Table 1 and Fig. 1), which were discriminative for all three species, neither *C. bracarensis* nor *C. nivariensis* were identified among our strains. *Candida tropicalis*, considered to be one of the three most frequent NACs, is isolated from invasive *Candida* infections, especially candiduria and candidemia [1,22]. We found this species as the second most prevalent agent isolated from urine and blood samples. Superficial infections of the nail and skin due to *C. tropicalis* are rarely seen [23] but in our study more than 75% of the *C. tropicalis* strains were isolated from onychomycosis. Like other NACs, most of the *C. kefyr* strains (55%) of the study were isolated from nail infection. *Candida guilliermondii* is an infrequent species worldwide with few reports in the literature regarding epidemiology and prevalence of the fungus in clinical settings. However, similar to the study of Kiraz and Oz [3], the species had the lowest rate of isolation (0.6%) in the present study and all five strains were recovered from onychomycosis.

Generally, as most of the clinical specimens in the study (67%) were from nail and vagina, *C. albicans* was the most frequently isolated species, however, isolation of non-*C. albicans Candida* species exceeded *C. albicans* in systemic specimens like blood and urine.

In conclusion, we did not have a systematically designed program to collect our samples; however, molecular identification of yeast isolates could show a clue of the microbial epidemiology of pathogenic yeasts. This report described the first preliminary molecular epidemiologic survey of the species distribution profile of clinically important *Candida* species in Iranian patients. The simple, inexpensive, rapid and reliable PCR-RFLP system used in the study has the potential to identify most clinically isolated yeasts.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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This paper was first published online on Early Online on 6 March 2013.

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