Original Article

Identification of Medically Important *Candida* Species by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism Analysis of the rDNA ITS1 and ITS2 Regions

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Abstract

Aim: We aimed to identify the distribution of species in candidal strains isolated from clinical samples and restriction fragment length polymorphism (RFLP) method based on *Msp* I and *Bln* I restrictive enzyme cuts of polymerase chain reaction (PCR) products after the amplification of ITS1 and ITS2 regions of rDNA genotypically. **Materials and Methods:** One hundred and fifty candidal strains isolated from various clinical samples were studies/ included. Phenotypic species assessment was performed using automated VITEK-2 system and kit used with the biochemical tests. Common genomic region amplification peculiar to candidal strains was carried out using ITS1 and ITS2 primer pairs. After the amplification, PCR products were cut with *Msp* I and *Bln* I restriction enzymes for species identification. **Results:** The majority of *Candida* isolates were isolated from urine (78.6%) while other isolates were composed of strains isolated from swab, wound, blood and other samples by 11.3%, 3.3%, 2% and 4.7%, respectively. The result of RFLP analysis carried out with *Msp* I and *Bln* I restriction enzymes showed that candidal strains were *Candida albicans* by 45.3%, *Candida glabrata* by 19.3%, *Candida tropicalis* by 14.6%, *Candida parapsilosis* by 5.3%, *Candida krusei* by 5.3%, *Candida lusitaniae* by 0.6% and other candidal strains by 9.3%. **Conclusion:** When the ability to identify *Candida* to species level of phenotypic and PCR-RFLP methods was assessed, a great difference was found between these two methods. It may be argued that *Msp* I and *Bln* I restriction enzyme fragments can be used in the identification of medically important *Candida* species. Further studies are needed to develop this kind of restriction profile to be used in the identification of candidal strains.

Keywords: *Candida*, *Msp* I and *Bln* I, polymerase chain reaction, restriction fragment length polymorphism

Introduction

The *Candida* as a normal flora member of mucocutaneous tissue, gastrointestinal tract and genitourinary tract in healthy individuals are opportunistic pathogens.[1] In humans, *Candida* are one of the most important aetiologic groups of skin and mucosal infections and other systemic infections.[2-5] Systemic *Candida* infections are increasing significantly in HIV-infected persons; neutropenic individuals who take anticancer therapy are subjected to unconscious use of widespread antibiotics, metabolic diseases (such as diabetes mellitus) and some kinds of other disorders.[2-5]

It has been reported that the invasive infections due to the *Candida* species have significantly increased in recent years. According to the recent data, the *Candida* species are the fourth most important cause of the bloodstream infections. *Candida* cause both superficial infections of the skin and the mucosa and the profound infections which might cause even death. Severe *Candida* infections are deep, invasive, systemic, disseminated, haematogenous disseminated and haematogenous infections.[6-10]

Even though other *Candida* species were isolated rarely in the past, it has been reported that 70%–90% of the agents were *Candida albicans* and 5% of them were *Candida glabrata* and *Candida tropicalis*. But now, the data show that apart from these species, which are detected commonly in the

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past, the prevalence of other species has been increasing recently. The *Candida* infection scale shows that patterns of the preponderance are now on the side of the non-albicans (*C. glabrata*, *C. tropicalis*, *Candida parapsilosis* and *Candida krusei*) instead of *C. albicans*. For example, it has been reported that *Candida glabrata* is the second most common agent of the vaginal candidiasis.[11-13]

In addition to, variation among epidemiological patterns, has also been reported that the resistance of the *Candida* species to conventional treatment is now increasing.[7,14] Since most of the hospitals have not enough facilities to detect other species of the *Candida*, they can only differentiate *C. albicans* and non-albicans. The routine diagnostic methods are usually limited to differentiate *Candida* species. This limitation is a result of either the lack of definitive diagnostic method or inability of the method. *Candida dubliniensis* was first identified in 1995 from clinical samples and has some phenotypic similarities (germ tube [+], generating Klamidospora forms) with *C. albicans* and is very difficult to be differentiated using standard diagnostic methods.[15] In the late 1990s, it was reported that the strains (*C. dubliniensis*) were susceptible to the antifungals, but in recent years, fluconazole-resistant strains and also multidrug resistance has been reported.[16-19]

Nowadays, most laboratories use routine diagnostic procedures to differentiate the strains whether they are *C. albicans* or non-albicans, and the choice of antifungal drug is made empirically. To determine the distribution of *Candida* species, it is necessary to know choice of the antifungal drugs according to the antifungal resistance patterns and the infection control procedures amongst the various health associations in different cities and countries. The distribution of *Candida* species and antifungal resistance patterns can vary between cities and even between the health associations in the same city. Knowledge about the distribution of *Candida* species and the drug resistance patterns is very important to prevent the spread of multidrug-resistant strains and for effective treatment.[17,20]

It is observed that opportunistic *Candida* infections have increased significantly in recent years. The prevalence of *Candida* infections have increased particularly in immunosuppressive people like patients with HIV, cancer or through widespread use of immunosuppressive chemotherapy. The identification of *Candida* species – the cause of the disease – is of high importance because the pathogenicity and antifungal susceptibility of *Candida* differ according to species.[11]

With the current study, we aimed at identifying the distribution of Candida species isolated from clinical samples using an automatic system for phenotypic identification and restriction fragment length polymorphism (RFLP) method based on Msp I and Bln I restriction digestion of ITS1 and ITS2 region PCR amplicons.

Materials and Methods

Collecting the samples and microbiologic culture

The samples (urine, wound swab, vaginal swab, sputum, bronchoalveolar lavage fluid, catheters, blood, thoracocentesis fluid and sperma) were taken from the patients who received treatment in several polyclinics and departments of Mustafa Kemal University Medical School, Health Practice and Research Hospital and were analysed in microbiology laboratory. Origins of the Strains were isolated from urine samples (79%), vaginal swap samples (11%), wound samples (3%), blood samples (2%), bronchoalveolar lavage samples (1%), catheter samples (1%), sputum samples (1%), thoracentesis samples (1%) and sperma samples (1%). The samples which were collected from different departments were inoculated onto Sabouraud dextrose agar. The plates were incubated at 37°C for 48 h. Colony count $\geq 10^5$ cfu/ml in urine cultures and pure or predominant growth in other cultures were evaluated as pathogenicity criteria. Germ tube-positive strains were microbiologically considered as *C. albicans*. Others (non-albicans) were analysed with automatised system (VITEK-2, bioMerieux, France) and kits used for biochemical tests. Moreover, antifungal susceptibility of the isolated candidal strains was measured by AST-YS06 (bioMerieux, France) kit as recommended by commercial company.

The *C. albicans* (ATCC 90028), *C. krusei* (ATCC 6258), *C. parapsilosis* (ATCC 22019), *C. tropicalis* (ATCC 22019) and *C. glabrata* (ATCC 32554) strains were used as standard strains for the aim of quality control. In accordance with the Clinical and Laboratory Standards Institute criteria, the minimum inhibitory concentration (MIC) value of antifungal drugs for the *Candida* species was evaluated as susceptible, intermediate and resistant. Since the MIC value of the amphotericin B was not standard, the value MIC ≥2 mg/l was taken as resistant (R) (National Committee for Clinical Standards 2002).[15]

Phenotypically identified strains were reserved for PCR-RFLP analyses. For these analyses, first genomic DNA extraction and PCR amplification of the strains were performed, and after restriction of the amplification products by *Msp* I and *Bln* I enzymes, restriction fragment analyses allowed genotypic identification.

Genomic DNA extraction and polymerase chain reaction amplification

Commercially provided ZR Fungal/Bacterial DNA Kit (Zymo Research) was used for genomic DNA extraction.

PCR amplification was performed using ITS1 and ITS4 primers from the isolated genomic DNA samples. PCR mix was prepared before this process. Accordingly, each of the Eppendorf tube contained 36.8 µl distilled water, 5 µl buffer, 1 µl dNTP, 0.2 µl Taq DNA polymerase, 3 µl $MgCl_2$, 1 µl forward primer and 1 µl reverse primer for each patient sample. It was vortexed and after 2 µl genomic DNA was added. A total

of 50 µl volume was obtained and the amplification cycle was performed. The PCR procedure was as follows: initial denaturation step at 94°C for 5 min followed by 35 cycles consisting of denaturation (94°C for 1 min), annealing (56°C for 1 min) and extension (72°C for 1 min), followed by a final extension step at 72°C for 7 min.

Scanning the polymerase chain reaction products with agarose gel electrophoresis method

After PCR process, the existence of the amplification products was detected by running them in agarose gel (2%) and by screening the bands through Wealtec, Dolphin-View, USA, screening device.

Restriction fragment length polymorphism

The PCR products, which were obtained after amplification, were cut by *Msp* I and *Bln* I restriction enzymes for RFLP analyses. The target area of these enzymes on the DNA molecules were as follows: the enzyme *Msp* I recognised and cleaved the sequence CCGG while *Bln* I recognised the sequence CCTAGG.[21]

Restriction enzyme analyses with *Msp* I and *Bln* I

A total volume of 25 µl with PCR amplification product and *Msp* I/*Bln* I was prepared as follows: digestion reactions were performed for each of the selected restriction enzymes (*Msp* I and *Bln* I) in a total volume of 25 µl containing 5 µl of × 10 reaction buffer, 1 µl of each restriction enzyme (10 U/µl), 10 µl of the PCR products of each sample and 9 µl of distilled water. Restriction process was done at 37°C and after 4 h incubation.

Analyses of restricted products

The control of the restricted products was made in agarose gel with 3% concentration. Ten microlitres of PCR products which were cut by *Msp* I and *Bln* I and 3 µl loading dye were mixed and loaded into gel. Restricted products were run with 100 bp DNA ladder and then screened by ultraviolet transilluminator (Wealtec, Dolphin-View, USA) and checked.

Results

In the study, the distribution of strains of Candida according to the departments was as follows: 33 (22%) isolates from the internal diseases Intensive Care Unit, 27 (18%) isolates from the department of gynaecology and obstetrics, 19 (12.6%) isolates from the department of internal diseases, 18 (12%) isolates from the surgery Intensive Care Unit, 11 (7.3%) isolates from the department of urology, 8 (5.3%) isolates from the department of brain and nerve surgery, 6 (4%) isolates from the department of chest diseases, 5 (3.3%) isolates from paediatric department, 4 (2.6%) isolates from the department of internal diseases – endocrinology, orthopaedics and traumatology, infectious diseases and dermatology, 2 (1.3%) isolates from the coroner Intensive Care Unit and physical therapy and rehabilitation and 1 (0.6%) isolate from the department of cardiology, paediatric neurology and eye diseases.

According to the phenotypic identification, the distribution of isolates were found to be as 48.6% (73/150) for *C. albicans*, 17.3% (26/150) for *C. tropicalis*, 17.3% (26/150) for *C. glabrata*, 4.0% (6/150) for *C. parapsilosis*, 4.0% (6/150) for *C. krusei*, 0.6% (1/150) for *Candida lusitaniae*, 0.6% (1/150) for *Candida kefyr*, 0.6% (1/150) for *Candida famata*, 0.6% (1/150) for *Candida lipolytica* and 6% (9/150) for others [Figures 1-3].

According to the genotypic identification, the distribution of species were as follows: *C. albicans* 45.3% (68/150), *C. glabrata* 19.3% (29/150), *C. tropicalis* 14.6% (22/150), *C. parapsilosis* 5.3% (8/150), *C. krusei* 5.3% (8/150), *C. lusitaniae* 0.6% (1/150) and (9.3%; 14/150) others [Figures 1-3].

According to the genotypic identification of the strains, the distribution of isolates according to departments, is stated below: of 33 *Candida* spp. which were isolated from internal diseases Intensive Care Unit, 15 (45.4%) were *C. albicans*, 5 (15.1%) were *C. tropicalis*, 5 (15.1%) were *C. glabrata* and 3 (9.09%) were *C. parapsilosis*. When the distribution of species that were obtained from gynaecology and obstetrics department which provided the second most common isolates following internal diseases Intensive Care Unit was examined (a total of 27 isolates), strains isolated at the highest ratio were *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. krusei* and *C. parapsilosis*; 14 (51.8%), 5 (18.5%), 3 (11.1%), 2 (7.4%) and 2 (7.4%), respectively. The distribution of species obtained from internal diseases department where a total of 19 candidal strains were isolated, was as follows: *C. albicans* 47.3% (9/19), *C. tropicalis* 21.05% (4/19) and *C. glabrata* 15.7% (3/19). The distribution of species identified genotypically according to departments is shown in Table 1.

In the study, *C. albicans* was the most commonly isolated species, but there was a discrepancy of 5.5% between conventional (phenotypic) and PCR-RFLP methods. Similarly, there were also discrepancies in the identification of *C. tropicalis*, *C. glabrata* and *C. parapsilosis* by 21.4%, 16.0% and 33.0%, respectively [Table 2].

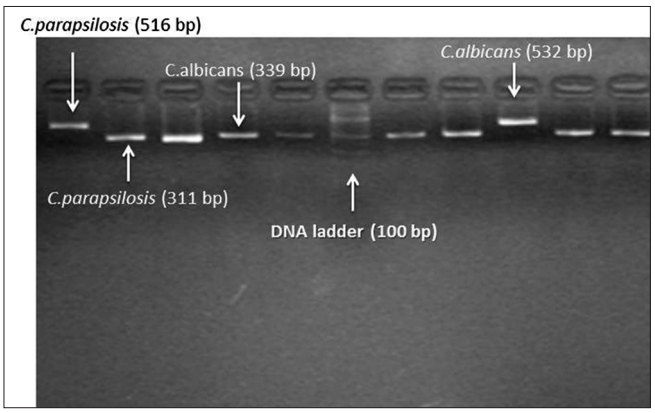

Figure 1: Some polymerase chain reaction-restriction fragment length polymorphism amplified products by *Msp* I restriction enzyme from the strains of *Candida* species. Lanes 6: 100 bp DNA ladder. *Candida albicans*: Lanes 4, 8, 10, 11 (339 bp) and lane 9 (532 bp). C. *parapsilosis*: Lane 2, 3, 5, 7 (311 bp) and 1 (516 bp)

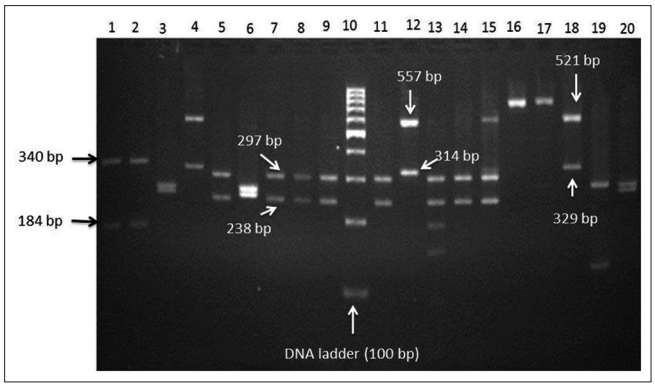

Figure 2: Some polymerase chain reaction-restriction fragment length polymorphism amplified products by *Msp* I restriction enzyme from the strains of *Candida* species. Lanes 10: 100 bp DNA ladder. *Candida albicans* (238; 297 bp), *Candida tropicalis* (329; 521 bp), *Candida glabrata* (314; 557 bp), *Candida tropicalis* (184, 340 bp)

When distribution of species obtained genotypically with the use of restriction enzymes *Msp* I and *Bln* I was examined, it was observed that *C. albicans* was 45.3% and *C. glabrata* was 19.3% while *C. parapsilosis* and *C. krusei* were less prevalent with a ratio of 5.3% both, which meant that RFLP method was not able to identify 10% of the isolates to species level [Table 3].

When distribution of species according to the clinical origins of isolates was examined, RFLP analyses revealed that 47.5% of *Candida* was *C. albicans* (56/118), 19.4% was *C. glabrata* (23/118), 14.4% was *C. tropicalis* (17/118), 4.2% was *C. krusei* (5/118), 3.3% was *C. parapsilosis* (4/118) and 0.8% was *C. lusitaniae* (1/118) in urine samples and 10.1% (12/118) of *Candida* could not be detected to species level. Again, in vaginal swabs where strains were isolated most after urine samples, *C. albicans* was the second most found *Candida* by 43.8% (7/16), *C. tropicalis and C. glabrata* were of 18.7% (3/16), *C. parapsilosis* of 12.5% (2/16) and *C. krusei* of 6.2% (1/16). Only 1 vaginal sample (6.2%) could not be characterised to species level. The distribution of species according to the clinical samples is shown in Table 4.

Amphotericin B, fluconazole and voriconazole antifungal susceptibility patterns of *Candida* species which were isolated from 37 patients who received a treatment from different departments were assessed. Table 5 demonstrated the MIC values of *Candida* species. Fluconazole resistance in one isolate each of *C. albicans* and one *C. glabrata* strains can be clearly seen in Table 5.

PCR amplification of 150 clinical samples was performed using ITS1 and ITS4 primers in the study. In the study, the restriction products which were exposed to *Msp* I restriction enzyme between the size of 261 bp and 557 bp were evaluated.[20] After the analyses of the restriction products, 68 *C. albicans* species (45.3%), 29 *C. glabrata* species (19.3%), 22 *C. tropicalis* species (14.6%), 8 *C. parapsilosis* species (5.3%), 8 *C. krusei* species (5.3%) and 1 *C. lusitaniae* species (0.6%) were found. Other strains were accepted as *Candida* spp.

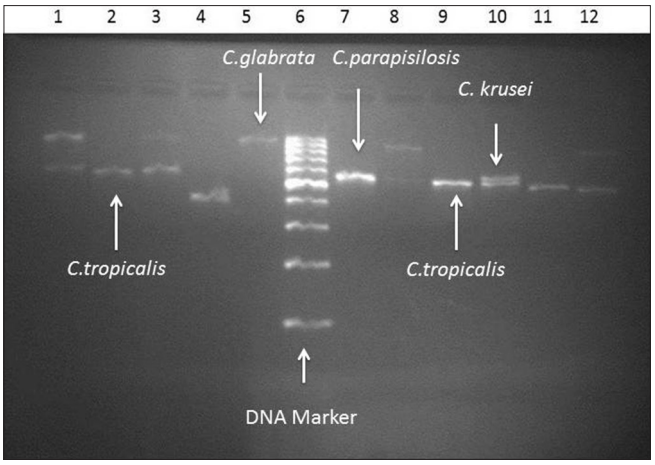

Figure 3: Some polymerase chain reaction-restriction fragment length polymorphism amplified products by *Bln* I restriction enzyme from the strains of *Candida* species. Lanes 6: 100 bp DNA ladder. *Candida glabrata*: 5, *Candida krusei*: 10, *Candida tropicalis*: 2 ve 9, C. *parapsilosis*: 7, *Candida* spp.: 1, 3, 4, 8, 11 ve 12

The analyses of restriction products using *Bln* I enzyme revealed a total of 82 non-albicans *Candida*. At the end of the restriction process with *Bln* I enzyme, 29 *C. glabrata* (35.5%), 22 *C. tropicalis* (26.8%), 8 *C. parapsilosis* (9.75%), 8 *C. krusei* (9.75%) and 1 *C. lusitaniae* isolates were determined. Other strains (14/82; 17.07%) were evaluated as *Candida* spp.

Discussion

With severe infections and having antifungal susceptibility differences, identification of pathogen *Candida* has a significant importance.[3,7] Through this study, we aimed to identify the distribution of Candida species isolated from clinical samples using an automatic system for phenotypic identification and RFLP method based on Msp I and Bln I restriction digestion of ITS1 and ITS2 region PCR amplicons. Furthermore, we aimed to determine the antifungal susceptibility of *Candida* to drugs which are routinely used (amphotericin B, flucytosine, fluconazole and voriconazole).

Candida infections are reported as the third main cause of the infections which occur in Intensive Care Units and fourth main cause of the bloodstream infections. *Candida* infections are very important as they sense that they prolong hospital stay and increase the morbidity and mortality. The most common species of *Candida* is *C. albicans* which is isolated from both healthy individuals and patients.[22,23]

The studies carried out over the last two decades have revealed that >80% of all forms of human candidiasis isolates were *C. albicans*. On the other hand, particularly in recent years, the infections due to other non-albicans *Candida* have been to be on the rise.[24,25]

It has been reported that the significant increase of non-albicans *Candida* as a pathogenesis of human candidiasis might be related with the development of diagnostic methods. The best example of this is that the CHROMagar chromogenic

Table 1: The distribution of *Candida* species which were identified genotypically according to departments

Strains	Departments															Total
	IDICU	Gynaecology and obstetrics	Internal diseases	SICU	Urology	Brain and nerve surgery	Chest diseases	Paediatrics	Orthopaedics and traumatology	Infectious diseases	Dermatology	Endocrinology	PTR	CICU	Cardiology	Paediatric neurology	Eye diseases	
Candida albicans	15	14	9	4	5	5	2	2	3	2	1	2	1	2	1	-	1	72
Candida glabrata	5	5	3	7	2	1	-	1	1	-	2	-	-	-	-	1	-	25
Candida tropicalis	5	3	4	3	1	1	1	1	-	1	-	1	1	-	-	-	-	28
Candida parapsilosis	3	2	-	1	-	1	-	-	-	1	-	-	-	-	-	-	-	6
Candida krusei	2	2	-	2	2	-	-	-	-	-	-	-	-	-	-	-	-	6
Candida lusitaniae	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	1
Candida spp.	3	1	3	1	-	-	3	1	-	-	1	1	-	-	-	-	-	14

IDICU: Internal Diseases Intensive Care Unit, SICU: Surgery Intensive Care Unit, PTR: Physical therapy and rehabilitation, CICU: Coroner Intensive Care Unit

media are at least as successful as other routine molecular methods in identifying *Candida* species which are isolated from fungaemia cases.[26]

Identification of etiological agent and antifungal drug choice is crucial for *Candida* infections. Unfortunately, antifungal susceptibility tests are not available in many healthcare centres today. Identification of intrinsically resistant strains or strains which are highly resistant to many antifungals is mainly advisor to empiric treatment. When compared with *C. albicans*, non-albicans *Candida* are more resistant to antifungals, and therefore, it is estimated that they are isolated more common in *Candida* infections recently.[22]

In this study, the urine samples were the ones from which *Candida* were isolated most (78.6%), followed by strains isolated from vaginal swabs (11.3%) and wound swabs (3.3%), respectively. It has been reported that the candiduria is more prevalent in inmates. But recently, with the increasing risk factors as a pathogen, candiduria is isolated more.[27]

The risk factors of candiduria include use of long-term broad-spectrum antibiotics, long duration of hospital stay, metabolic diseases and immunosuppressive treatments. In a study, it was reported that 21.3% of catheter related infections are caused by *Candida*.[28,29]

Catheter is a common route for pathogens to enter urinary tract, and long-term catheterisation can cause colonisation of microorganisms. According to a study of a patient with candidiasis, *C. albicans* was the most common isolate (51%) and *C. glabrata* was the second most common isolate by 16%.[30]

In a study which included 250 catheterised patients, both phenotypic and genotypic analyses were performed to determine the cause of candiduria to detect candiduria prevalence. To identify the yeasts, Firstly, to identify the yeasts, phenotypic methods such as germ tube method, hypha and pseudohypha or chlamydospore growth on CMA + TW80 and CHROMagar *Candida* were performed and evaluated. Genomic DNA of all species was first analysed with PCR and then RFLP methods employed. As a result of urine analyses of 95 females and 145 males, *Candida* spp. was isolated from 40 samples. The prevalence of candiduria was estimated as 16%.[31] In our study, it is clear that the distribution of species by identification of isolates based on PCR-RFLP concurred with literature.[31]

We isolated 57 *C. albicans* from 118 urine samples (48.3%). The second most isolated species was *C. glabrata* and the third was *C. tropicalis* by 19.4% and 14.4%, respectively. As in the urinary samples, *C. albicans* was again the most isolated species from vaginal swabs (37.5%; 6/16) with *C. tropicalis* and *C. glabrata* followed by 18.7% (3/16) both. Apart from these species, *C. parapsilosis* (12.5%) and *C. krusei* (6.2%) were isolated from vaginal samples.

The range and the distribution of pathogen *Candida* depend on anatomical localization and environmental conditions.

Table 2: The difference between phenotypical and genotypical identification methods

Species	Phenotypic method (*n*)	Genotypic method (*n*)	Difference, *n* (%)
Candida albicans	72	68	4 (5.5)
Candida tropicalis	28	22	6 (21.4)
Candida glabrata	25	29	4 (16)
Candida parapsilosis	6	8	2 (33)
Candida krusei	6	8	2 (33)
Candida lusitaniae	1	1	-
Candida kefyr	1	-	1 (100)
Candida lipolytica	1	-	1 (100)
Candida famata	1	-	1 (100)
Candida spp.	9	14	5 (55.5)

Table 3: The distribution of *Candida* isolates obtained genotypically using the *Msp* I and *Bln* I enzymes

Strains	*n* (%)
Candida albicans	68 (45.3)
Candida glabrata	29 (19.3)
Candida tropicalis	22 (14.6)
Candida parapsilosis	8 (5.3)
Candida krusei	8 (5.3)
Candida lusitaniae	1 (0.6)
Candida spp.	14 (9.3)

Non-albicans *Candida* are mostly isolated from throat and vagina.[32]

The choice of antifungal drug is made according to clinical condition, infected area, pharmacodynamics and pharmacokinetics. Fluconazole is an antifungal drug and the best choice for urinary tract infections and also affects many of *Candida*. In concordance with literature, in our study, non-albicans *Candida* were more resistant to antifungals than *C. albicans*.[33]

Many methods are available to identify *Candida* species. These methods can be divided into two main groups: phenotypic and genotypic methods. In our study, we characterized the isolated *Candida* from the clinical samples using both phenotypic method and genotypic PCR-RFLP method. According to phenotypic method, *C. albicans* was the most isolated species (48.6%). *C. tropicalis* and *C. glabrata* were isolated with a same ratio of 17.3%. Moreover, the phenotypic method revealed 6 *C. parapsilosis*, 6 *C. krusei*, 1 *C. lusitaniae*, 1 *C. kefyr*, *C. famata* and 1 *C. lipolytica*. Nine out of 150 (6%) could not be identified with phenotypic methods. When we looked at the distribution of species in strains identified genotypically, 45.3% of the strains were *C. albicans*, 19.3% were *C. glabrata*, 14.6% were *C. tropicalis,* 5.3% were *C. parapsilosis,* 5.3% were *C. krusei* and 0.6% were *C. lusitaniae*. Fourteen of 150 isolates (9.3%) could not be identified.

When these two methods were compared, 4 (5.6%) of the 72 samples which were identified as *C. albicans* with phenotypic method were not *C. albicans* according to genotypic method. This discrepancy amongst *C. albicans* was clearer and more evident amongst other non-albicans *Candida*. For example, 21.4% (22/28) of the 28 isolates identified as *C. tropicalis* with phenotypic method were not detected as *C. tropicalis*. Likewise, a total of 25 *C. glabrata* were identified with phenotypic method while 29 *C. glabrata* were identified with genotypic methods. The highest discrepancy of the methods used for identifying the species occurred between *C. parapsilosis* and *C. krusei* by 33.3%. Since these two species are known to be resistant to antifungals, the importance of true and fast identification is evident.

In our study, there were also similar discrepancies between these two methods in terms of identifying rarely isolated species. For example, one *C. kefyr*, one *C. lipolytica* and one *C. famata* isolates were phenotypically identified, but none of them was verified as *C. kefyr*, *C. lipolytica* or *C. famata* genotypically. The only consistence between these methods was seen in identifying *C. lusitaniae* phenotypically and genotypically.

Furthermore, 6% of all isolates could not be identified phenotypically whereas 9.3% of all strains could not be identified genotypically. We are of the opinion that we may be able to identify the isolates which are genotypically non-identified by increasing the number of restriction enzymes, which thus increases the sensitivity.

C. albicans is the main cause of most of *Candida* infections, but it has been reported that the prevalence of *C. glabrata* and *C. krusei*, more resistant to azoles, has significantly increased recently.[34]

The early diagnosis of invasive fungal infections is highly important to lower the rate of mortality. Though new molecular methods were reported recently, there is always a demand for a simple, fast and cost-effective method. In a study, the region ITS1-5.8S-ITS2 (510-879 bp) of rDNA was amplified by ITS1 and ITS4 primers, RFLP was then performed and thus the most common 6 species of *Candida* were identified.[35]

In our study, ITS1 and ITS2 regions were amplified only using *Msp* I and *Bln* I restriction enzymes, 6 species of *Candida* isolated from *Candida* infections were successfully identified.

Table 4: The distribution of *Candida* species genotypically identified according to the clinical samples

Type of samples	*Candida albicans*	*Candida glabrata*	*Candida tropicalis*	*Candida parapsilosis*	*Candida krusei*	*Candida lusitaniae*	*Candida* spp.
Urine	56	23	17	4	5	1	12
Vaginal swab	7	3	3	2	1	-	1
Wound	2	1	-	-	1	-	1
Blood	-	-	1	2	-	-	-
Bronchoalveolar lavage fluid	1	-	1	-	-	-	-
Catheter	2	-	-	-	-	-	-
Sputum	-	1	-	-	-	-	-
Thoracocentesis fluid	-	-	-	-	1	-	-
Sperma	-	1	-	-	-	-	-
Total, *n* (%)	68 (45.3)	29 (19.3)	22 (14.6)	8 (5.3)	8 (5.3)	1 (0.6)	14 (9.3)

Table 5: The antifungal susceptibility patterns of the isolates

Candida (*n*)	Antifungal drugs	MIC value (mg/L)										
		≥64	32	16	8	4	2	1	0.5	≤0.25	≤1	≤0.12
Candida albicans (21)	Amphotericin B							13	7	1		
	Flucytosine										21	
	Fluconazole			1	2						18	
	Voriconazole											21
Candida glabrata (5)	Amphotericin B							2	3			
	Flucytosine										5	
	Fluconazole		1			1					3	
	Voriconazole					1						4
Candida tropicalis (4)	Amphotericin B						1	1	2			
	Flucytosine										4	
	Fluconazole	1				1	1				1	
	Voriconazole											4
Candida parapsilosis (3)	Amphotericin B						1		2			
	Flucytosine										3	
	Fluconazole					1					2	
	Voriconazole											3
Candida lusitaniae (1)	Amphotericin B								1			
	Flucytosine										1	
	Fluconazole										1	
	Voriconazole											1
Candida spp. (3)	Amphotericin B							1		2		
	Flucytosine										3	
	Fluconazole										3	
	Voriconazole											3

MIC: Minimum inhibitory concentration

Conclusion

In the study, restriction of ITS amplification products with *Msp* I enzyme predicted the specific patterns of each species. Moreover, PCR-RFLP analyses of the clinical isolates were compared with the standard species. Similar patterns were obtained between *C. albicans* and *C. dubliniensis* when restricted by *Msp* I. Therefore, the use of other restriction enzymes was necessary. We recommend PCR-RFLP because it is a simple and easy method to identify the *Candida* in medical mycology laboratories.

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Conflicts of interest

There are no conflicts of interest.

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