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Genotyping of Candiduria Pathogens by Multiple Molecular Markers

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ABSTRACT

PURPOSE: Urinary tract infection (UTI) is considered one of the major diseases affecting humans. The aim of the present study was to diagnose fungal species associated with urine of patients undergo urinary tract infections, genetically using multiple markers and phenotypically in Iraq. METHODS: A total of 150 urine specimens were collected from 150 UTI suspects patients from October 2012 to October 2013. Fungal organisms were isolated and identified based on morphological characters and colony color on CHROMagar, genetically typing 18 *Candida* isolates were performed with specific primer pairs, four sets of universal primer pairs were used for amplifying the target regions in rDNA genes, and analysis of ALT repeats in the *Candida* isolates. RESULTS: We isolated 25 filamentous fungal species including *Acremonium polychromum*, *Penicillium digitatum*, *Aspergillus fumigatus*, and *Asp. niger*. Thirty *Candida* isolates were classified phenotypically and genotyping of ITS regions showing polymorphic PCR products into four *Candida* species: *C. albicans*, *C. famata*, *C. glabrata* and *C. guilliermondii*. 14 isolates of *C. albicans* were identification of, while the primer pairs ITS1/LR3 and ITS3/LR3 were elucidated unique genotypes. The LR12/5SRNA showed variations in the lengths of the IGS1 region, and showed variations in the numbers of 5RNA among the *Candida* isolates of interest, while the ASDCF/PCSCR produced multiple genotyping patterns. CONCLUSIONS: Our conclusions suggest the importance of the role of *C. albicans* in UTI based on the other companions in the UTI samples. On the other hand, the molecular assays for identification of yeast are powerful tools for the diagnosis and genotyping of *Candida.*

INTRODUCTION

Candiduria has been uncommon and largely ignored disease, and several unresolved complicated urinary tract infections (UTIs) continue to remain, especially due to lack of a reliable diagnostic method of the *Candida* pathogen [1]. Filamentous fungi and *Candida* spp. are one of the important pathogens that cause urinary tract (UT) failure, and candiduria is rarely encountered in otherwise healthy individuals with normal UTs [2]. Earlier studies referred to the role of *Candida* spp. in UTIs. Epidemiologically, Ang *et al.* [3] reported that 26.5% of all UTIs are caused by *Candida* species.

The most common yeasts causing complicated and uncomplicated UTIs are *Candida albicans* and *C. glabrata*. UTI complications are common in hospitalized patients [4]. Relevant infection to these devices occurs in 1–60% of patients. It was reported that *Candida* spp. cause 20% infections. Infections associated with the use of medical devices caused >50% of all hospital infections [5]. Moreover, approximately one-third of hospitalized patients with urine cultures showed positive tests [6]. In hospitalized patients, diseases such as diabetes may predispose patients to fungal candiduria by predisposing them to *Candida* colonization of the vulvovestibular area by enhancing urinary fungal growth in the presence of glycosuria [7].

Sobel *et al.* [8] reported that *C. albicans* represent the common causative agent of mucosal yeast infections (70%) in HIV. Several species of *Candida* including *C. albicans*, *C. glabrata*, and *C. guilliermondii* are suspected as common human pathogens. Most UT patients suffering from fungal infections and disseminated these infections if considered the fungal presence is only due to normal colonization [9]. Therefore, a precise assay to diagnosis UT infectious agents can reduce the risk of complications in patients. Phenotypic assays for fungal diagnosis have the limitations of being time consuming, less precise, and with more risk for contamination. On the other hand, molecular assays are more reliable methods for screening microbial pathogens, especially for patient suffering from complicated infections, and they also reduce the suffering and provide precise diagnosis for deciding the treatment [10].

Dennis Lo [11] reported the application of molecular assay on urine samples directly for the extraction of DNA. For molecular diagnosis, these results suggested the exciting possibility that the noninvasive diagnosis and monitoring of several diseases may be achieved by analyzing a body fluid type that can be obtained safely and in virtually unlimited amounts.

The aim of the present study was to evaluate precise and reliable molecular markers for the diagnosis different *Candida* spp. Companions of UTI, especially for patients with UT failure, and detected the risk yeast suspected with the UTI complication based on phenotypic and molecular assays.

METHODS

Collection of Urine Samples

A total of 150 urine samples were collected from patients suffering from UTIs and renal failure from the Recovery Center Unit of Kidney in the Marjan hospital, Babylon province. The patients included 63 men and 87 women, aged 10–80 years and sampling was performed between 2012 and 2013.

Briefly, 0.5 mL of suspected urine sample was cultured on Sabouraud's agar medium by powering methods and incubated at 30°C for 24–48 h for yeast and up to 7 days for filamentous fungi isolation.

Culturing on CHROMagar Candida

Chromogenic medium contains chromogenic substrates that react with enzymes secreted by the target microorganisms to yield colonies of varying colors. CHROMagar Candida differential agar is a rapid, selective and differential medium for *Candida* spp. [12].

Extraction of genomic DNA

Genomic DNA of 18 isolates of *Candida* spp. were subjected to DNA extraction and PCR assays. Yeasts were individually grown on Sabouraud's dextrose agar and incubated at 30°C for 2 days. Part of *Candida* colony was picked and suspended in 400 µL of lysis buffer (400 mM Tris-HCl, 20 mM EDTA, 150 mM NaCl, and 0.5% SDS; pH 8.5), incubated for 10 min and vortexed for 2 min. Then, 150 µL of phenol-chloroform was added and the mixture was vortexed again for 2 min and centrifuged at 5000 rpm for 2 min; the supernatant was decanted into fresh sterile tubes, to which 500 µL of isopropanol was added gently for 1 min and centrifuged at 12000 rpm for 12 min. The pellet was washed with 70% ethanol and centrifuged at 5000 rpm for 2 min, dried, suspended in 75 µL elution buffer, and preserved at −20°C until use [13].

Primers and PCR Assays

The rDNA was amplified by using the polymerase chain reaction (PCR) technique in which four sets of universal primers pairs (ITS1/ITS4, ITS1/LR3, ITS3/LR3 and L12/5SRNA) was used (Table 1, Fig. 1), while the ALT repeat was amplified with a primer pair of ASDCF and PCSCR. All treatments were performed separately in a single reaction mixture. The PCR mixture was amplified by the Thermal Cycler PCR System (Labnet, USA). Different PCR conditions were designated for each primer pair. The PCR conditions for most primers set were 95°C for 3 min, followed by 30 cycles of 94°C for 30 s, annealing at 56°C for 40 s, extension at 72°C for 1.10 min, and a final extension at 72°C for 10 min except the annealing temperatures for ASDCF/ PCSCR 30 s at 58°C, and LR12/ 5SRNA at 55°C for 1 min.

The PCR products for each target region were run on 1.2% agarose gel (Bio Basic Canada Inc.) electrophoreses performed at 70 V. in TBE buffer. The gel was pre-stained with 0.05% ethidium bromide. The DNA bands were detected by Desktop Gel imager scope 21 ultraviolet transilluminator.

RESULTS

Culturing on CHROMagar Candida

On the CHROMagar Candida, 14 *Candida* isolates showed apple-green color and were classified as *C. albicans*, 4 isolates of non-albicans showed red- pink color spectrum and were classified as *C. famata*, *C. glabrata*, and *C. guilliermondii* (Table 2).

Molecular identification of *Candida* form genus with specific primers

A total of 18 *Candida* isolates were identified with the specific primer pair CAINBF and CAINBR that produced amplicon size of 210 bp (Table 2).

Molecular diagnosis for *C. albicans*

A total of 14 out of 18 *Candida* isolates were identified as *C. albicans* based on the specific primer pair (CABC). The amplification of the targeted region produced an amplicon size of 665 bp (Fig. 2, Table 2).

Genotyping ITS1-5.8S-ITS2 region of *Candida* spp.

The primer pair of ITS1/ITS4 successful amplified the ITS1-5.8S-ITS2 region and helped classify the 18 isolates of *Candida* into 4 genotypes groups: i) 14 isolates with an amplicon size of 530 bp classified as *C. albicans*, ii) 2 isolates classified as *C. glabrata*, and iii) and iv) one isolate each with a PCR product of 650 bp and 603 bp sizes were classified as *C. famata* and C. *guilliermondii*, respectively (Table 2).

Genotyping of the ITS1-5.8S-ITS2 region and part of the 25S region of *Candida* spp.

The primer pair of ITS1/LR3 successful amplified the ITS1-5.8S-ITS2 region and a part of the 25S region for 18 isolates of *Candida* producing unique genotypes; isolates 1-3, 5-7, 9-10, 12, 14, 15 and 18 showed homologues pattern; isolates 4, 11, 13, 16, and 17 had the same genotype, isolate 8 belonged to a unique genotype (Fig.3, Table 2). This data has been recorded for the first time, but the amplicons size coincidence with the amplicons of the ITS1-5.8S-ITS2 region with extra length based on the complementary site of LR3 primer on rDNA.

Genotyping of the targeted region of the primer pair ITS3/LR3 for 18 *Candida* isolates

The primer pair ITS3/LR3 successful amplified the 5.8S-ITS2 region and part of the 25S region for18 *Candida* isolates producing unique genotypes (700–1100 bp); isolates 1, 2, 5, 7, 9, 14, and 15 were homologous; isolates 3, 4, 6, 10–13, and 16–18 belonged to the same genotype; and isolate 8 belonged to unique genotype (700 bp) (Fig. 4; Table 2). This data has been recorded for the first time, but the amplicon size coincide with amplicons of the 5.8S-ITS2 region with extra length due to the site of LR3 primer site on rDNA.

Genotyping of the IGS1 region by the primer pair LR12 /5SRNA for 18 *Candida* isolates

The primer pair LR12/5SRNA successful amplified the IGS1 region for18 *Candida* isolates producing unique genotypes with single and double bands (Fig. 5; Table 2). The explanation for these variations correlates with the length different in this region, and there is more than one 5SR region in the IGS1 region.

Genotyping of ALT repeat of 18 *Candida* isolates.

The primer pair ASDCF/PCSCR successful amplified the ALT repeated region for18 *Candida* spp. producing unique genotypes with 750, 850 and 1100bp sizes; the isolates 2–9, 11–13, and

16–18 had homologous genotype with approximately 750, 900, and 1000 bp sizes; and isolates 10, 14, and 15 had homologous genotype with 850 bp size; isolate 1 had a unique genotype (750 bp size) (Fig. 6).

DISCUSSION

The results indicated that four *Candida* species suspect with UTI complications. We encountered problems in the application of recommended style with urine sampling include; how obligate the patients to bring particular volume of his or her urine, storage of samples for extended periods of time from clinics to laboratory. *Candida* isolates appear in a few colonies/plate in urine sample might correlate with the volume of urine sample 30 isolates of *Candida* were frequented in 150 patients (20%), this is low ratio but with risk consideration on human health. Our results showed present 14 isolates of *C. albicans* out of 30 isolates of *Candida* spp. these results were in agreement with those of Febre *et al.*[14] who reported that *C. albicans* with 46.15% frequency was the most abundant fungus, followed by *C. glabrata* (30.77%) from urine specimens. Their reports referred to appear Candida in urine as contamination, belief this idea create to quit Candiduria largely ignored disease. Many previous studies attempted identified *Candida* in urine by phenotypic and molecular assays [4, 15].

Phenotypic assay based on the CHROMagar candida did not accurate for identification. However, from the data described in Table-2 it is clear that non-albicans isolates shares many characteristics in their color properties on the CHROMagar. These results are in agreement with those of Ahmed *et al*. [10].

Molecular identification of *Candida* form genus with specific primers facilitated the diagnosis of *Candida* isolates and proves absent *Cryptococcus* sp. in urine samples under interest. This result is in concordance with that of Luo and Mitchell [16] and state the importance of concentration in PCR assays for the detection of *Candida* species.

As observed in the present study, the amplified ITS1–5.8S–ITS2 region and part of 25S region by set of primers pairs:ITS1/ITS4, ITS1/LR3 and ITS3/LR3 producing unique genotypes and proved to be adequate for the detection of different genotypes in 18 isolates under interest (Table 2 summarized the genetic patterns of *Candida* spp.).Our results of amplified the ITS1-5.8S-ITS2 region and helped classify the 18 isolates of *Candida* into 4 genotypes groups coincide with those of Fujita *et al.* [17]. The difference in the length of

some of the PCR products may be correlated with inter-evolution between these species This data(ITS1/LR3 and ITS3/LR3 PCR products) has been recorded for the first time, but the amplicons sizes coincidence with the amplicons of the ITS1-5.8S-ITS2 region in previous studies [18,19] .with extra band length based on the site of the primer LR3 on rDNA. Our results are based on used LR3 primer reproducible genetic variations among Candida species (Table 2).

The primer pair LR12/5SRNA successful amplified the IGS1 region. The explanation for these genetic variations may correlate with the length of this region, and there are more than 5SR region in the IGS1. These results are in agreement with those of Nguyen *et al.,* [20]. Primer pair ASDCF/PCSCR successful amplified the ALT repeated and generated many genotypes, these genotypes variable in tendum repeated number of *Candida* spp. These results are in agreement with those of Iwata *et al.* [13].

The present study is a pioneer in use of multiple molecular markers for analysis of genetic variability of *Candida* species those shared in UTI complications in Iraq, and this results will contribute greatly to the typing of main *Candida* species companion UTI patients in this country.

In conclusion, use of multiple markers is a precise molecular diagnostic tool for *Candida* spp., the common Candida especially *C. albicans* and *C. glabrata* in urine samples, which are highly suspected in cases of kidney failure and renal complications.

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Table 1: A list of primers used in the molecular assays

Primers	Sequence 5′-3′	NTs No.
CAINBF	(5′-GAGGGCAAGTCTGGTG-3′)	16
CAINB R	(5′-CCTGCTTTGAACACTCTAA-3′)	19
CABF59	5'-TTGAACATCTCCAGTTTCAAAGGT-3'	24
CADBR125	5'-AGCTAAATTCATAGCAGAAAGC-3'	22
ITS1	(5′-TCCGTAGGTGAACCTGCG-3′)	18
ITS3	(5′-GCATCGATGAAGAACGCAGC-3′)	20
ITS4	(5′-TCCTCCGCTTATTGATATGC-3′)	20
LR3	(5′-CCGTGTTTCAAGACGGG-3′)	17
ASDCF	(5′-ATGAACCACATGTGCTACAAAG-3′)	22
PCSCR	(5′-CGCCTCTATTGGTCGTCGAGCA-3′)	22
LR12	(5′-GAACGCCTCTAAGTCAGAATCC-3′)	22
5SRNA	(5′-ATCAGACGGGATGGGT-3′)	16

Table 2: Summarized characters the phenotypic and genotypic patterns of 18 *Candida* isolates

Isolate No.	Species name	CHROMagar	Amplicon sizes (bp) for primers set					
			CAIN	ITS	CABC	TS11/L	TS3/L3	IGS1
1	*C. glabrata*	red-pink	210	870		1400	980	1100
2	*C. albicans*	green	210	530	665	1200	900	1100
3	*C. albicans*	green	210	530	665	1200	1050	1100
4	*C. albicans*	green	210	530	665	1200	1100	1100
5	*C. albicans*	green	210	530	665	1200	1000	1100
6	*C. albicans*	green	210	530	665	1200	1100	1100
7	*C. glabrata*	red-pink	210	870		1400	1050	1100
8	*C. albicans*	green	210	530	665	1100	700	500+11
9	*C. albicans*	green	210	530	665	1200	1000	500+11
10	*C.*	red	210	603		1280	1100	500+11
11	*C. albicans*	green	210	530	665	1250	1100	1900+1
12	*C. albicans*	green	210	530		1250	1100	1900+1
13	*C. albicans*	green	210	530	665	1250	1050	900
14	*C. albicans*	green	210	530	665	1250	1000	1100
15	*C. famata*	pink	210	650		1350	1000	1100
16	*C. albicans*	green	210	530	665	1250	1050	1900+1
17	*C. albicans*	green	210	530	665	1250	1000	1100
18	*C. albicans*	green	210	530	665	1250	1000	1100

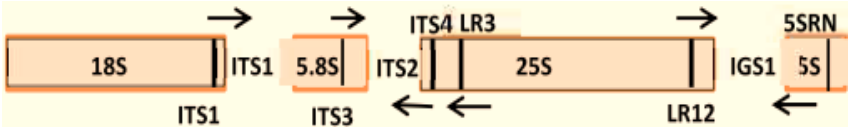

Fig.1: Locations of primers set targeting the rDNA genes in *Candida species* , primer pairs :ITS1/ITS4 for amplified ITS region, ITS1/LR3 for amplified ITS region with part 25S region ITS3/LR3 for amplified ITS2 region with part 25S region, LR12/5SRNA for amplified IGS1 region ,Primer site are marked as bold line. Arrows show primer direction

Fig.2: Gel electrophoresis of PCR products for 14 *C. albicans* isolates from urine samples. Lane 1–18 are of *Candida* isolates, M = molecular marker, 100 bp step

Fig.3: Gel electrophoresis of amplified targeted region of the primer pair ITS1/LR3 for 18 *Candida* spp. isolated from urine samples. Lanes 1–18 are of *Candida* isolates, M = molecular markers, 100 bp and 50 bp step

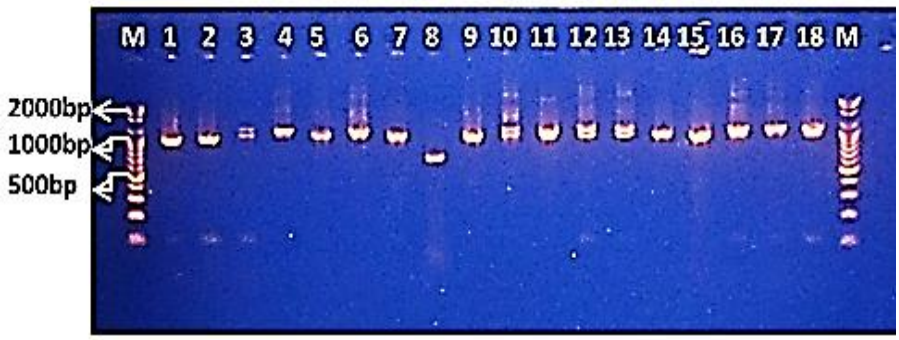

Fig.4: Gel electrophoresis of the amplified targeted region of the primer pair ITS3/LR3 for 18 *Candida* spp. isolated from urine samples. Lanes 1–18 are of *Candida* isolates, M = molecular marker, 100 bp step

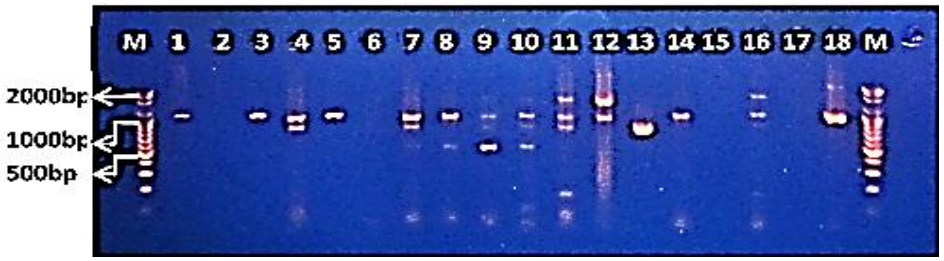

Fig.5: Gel electrophoresis of the amplified targeted IGS1 region of the primer pair LR12/5SRNA for 18 *Candida* spp. isolated from urine samples. Lanes 1–18 are of *Candida* isolates, M = molecular marker, 100 bp step

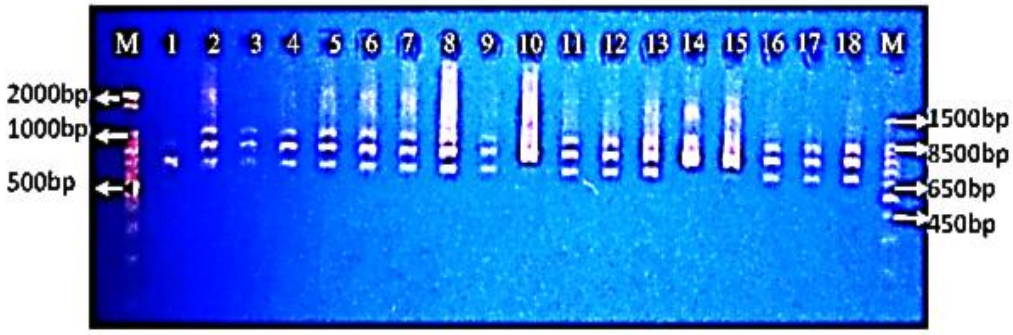

Fig.6: Gel electrophoresis of the amplified ALT repeated of 18 *Candida* spp. isolated from urine samples. Lanes 1–18 are of *Candida* isolates, M = molecular marker, 100 bp.**,**50bp step

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Table 1. A list of primers used in the molecular assays**.**

Table 2: Summarized characters the phenotypic and genotypic patterns of 18 *Candida* isolates.

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Fig. 5. Gel electrophoresis of the amplified targeted IGS1 region of the primer pair LR12/5SRNA for 18 *Candida* spp. isolated from urine samples. Lanes 1–18 are of *Candida* isolates, M = molecular marker, 100 bp step.

Fig. 6. Gel electrophoresis of the amplified ALT repeated of 18 *Candida* spp. isolated from urine samples. Lanes 1–18 are of *Candida* isolates, M = molecular marker, 100 bp.,50bp step.