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# IDENTIFICATION OF CANDIDA SPP. ISOLATED FROM URINE BY PHENOTYPIC METHODS AND MULTIPLEX PCR IN DUHOK, IRAQ

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**ABSTRACT:**
The present study was aimed to isolate and identify *Candida* species from urine samples obtained from patients with urinary tract infection from Duhok province. Species identification was based on colony color on chromogenic Candida agar as well as multiplex PCR method. Results revealed the prevalence of non-albicans *Candida*. *C.krusei* was the most frequently isolated species with prevalence of 26.3% followed by *C.kefyr* 23.68%, *C.glabrata* (18.42% and *C.albicans* 15.78%. Multiplex PCR method seemed to be more accurate and reliable for identification of *Candida* yeast.

**KEYWORDS:** Candida species, Multiplex PCR, Urine, Chromogenic Candida agar, Iraq.

## 1. INTRODUCTION

Detection of *Candida* species in urine of healthy people with normal urinary tract (asymptomatic) is rarely occurred. However, it is very common among hospitalized patients with urinary tract infections (Alvarez-Lerma *et al.*, 2003). The presence of fungi in the urine may be due to contamination, colonization of the catheter or infection (Fisher *et al.*, 1995). Candiduria is the main hospital infection that afflicts the urinary tracts (Kauffman, 2005). The most important risk factors which help emergence of candiduria are high utilization of antibiotics, long hospitalization periods, indwelling medical devices such as catheters, higher ages, diabetes mellitus, sexuality, gender and the use of immunosuppressive therapy (Bukhary, 2008, Behzadi *et al., 2015*).. *Candida* species are opportunistic mycoflora found frequently in genitourinary tract of humans especially in the immunocompromised, diabetics or pregnant women. If left untreated, may lead to systemic candidiasis, including disseminated disease, multiple organ failure or death (Hazen, 1995). The most common yeasts causing both complicated and uncomplicated UTI are *C.albicans* and *C. glabrata* (Bukhary, 2008). In a special condition *Candida* becomes an opportunistic human pathogen and includes: *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei*, *Candida guilliermondii*, *Candida lusitaniae*, *Candida kefyr*, and *Candida dubliniensis*, but they exhibits lower isolation rates, and there are also other species which are non-pathogenic (Ellis, 2007). Most UT patients suffering from fungal infections may disseminate these infections if considered that the fungal presence is only due to normal colonization (Zarei *et al*., 2009). Therefore, a precise assay to diagnosis UT infectious agents can reduce the risk of complications in patients (Nucci, 2000). Traditionally, the identification and classification of *Candida* species is time consuming and unreliable methods such as serotyping (Brawner, 1991), colony morphotyping (Soll, 1992), conventional culture techniques, and morphological and biochemical analysis (Williamson *et al*., 1986). Therefore, the improvement in molecular assay technology for identifying *Candida* species, such as randomly amplified polymorphic DNA analysis (RAPD), has overcome these limitations during the last couple of years. However, methods such as single and direct PCR or Multiplex PCR have not been used extensively despite being highly sensitive and specific with a shorter turn-around time (Jordan, 1994; Tietz *et al*., 1995; Chang *et al*., 2001; Fujita *et al*., 2001). Multiplex PCR is a rapid diagnostic assay which combines many specific species primers in one PCR tube. Hence, it could be used to identify more than one species in a specimen simultaneously (Luo and Mitchell, 2002). The aim of this study was directed to isolate and identify *Candida* species from urine of patients diagnosed of urinary tract infections attending Azadi hospital, Duhok province, Kurdistan Region, Iraq using Multiplex PCR method.

## 2. MATERIALS AND METHODS

### 2.1 Collection of samples

Urine samples were obtained from 283 patients (130 males and 153 females) attending the Azadi hospital, Maternity hospital, Central laboratory and private laboratories in Duhok city, Kurdistan region, Iraq during a period from October, 2013 to March 2014. Urine samples were collected in tightly closed sterilized and labeled plastic containers and brought to the laboratory for testing.

### 2.2 Isolation of yeasts

A drop from each urine sample was inoculated on Sabouraud's dextrose agar medium (SDA) (Lab. M Limited Co.Uk) amended with 0.250 mg/L Chloramphenicol for primary isolation of *Candida* species (Badiee *et al*., 2010). The plates were incubated at 37°C and examined after 24 to 48 hours.
Suspected *Candida* colonies with white to cream coloured were subcultured onto fresh SDA plates and incubated for 24-48 hours. For presumptive identification, *Candida* isolates were sub cultured onto chromogenic candida agar (Rapid labs Ltd,Essex,UK) which differentiate *Candida* species on the basis of colour change (Babic and Hukic, 2010). According to the manufacturer's instructions the colors of the colonies are as follows: *C. albicans* (Emerald, with metallic shine), *C.*

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*dublinensis* (Dark green, matt), *C. glabrata* (White, shiny), *C. krusei* (Light pink), *C. tropicalis* (Red purple).

### 2.3 DNA extraction and PCR amplification

Genomic DNA extraction and purification were performed using a Genomic DNA Extraction kit (provided by Jena Bioscience GmbH/ Germany) based on the guidelines.

For Multiplex PCR primer mixes were used, each primer pairs used were for a species of *Candida* they were grouped according the following criteria: (a) no primers in a group form dimers and /or interrupt PCR amplification ; (b) the number of the group should be as small as possible ;(c) each primer pairs yields one major PCR product, and each species of *Candida* should clearly be distinguished by the size (bp) of the PCR products, These primers were described by Kanbe *et al*. (2002) for sequencing of the *Candida* DNA topoisomerase II genes . In this study, three sets of primer groups were prepared. These groups were designated S1, S11 and S111, and referred to as a 'primer mix 'in this study. S1was composed of four specific primer pairs for identification of *C. albicans*, *C. parapsilosis* I, *C. parapsilosisII* and *C. guilliermondii*; S11was for *C. dubliniensis*, *C. krusei*, *C. kefyr* and *C. glabrata*; and S111 was for *C. tropicalisI*, *C. tropicalis II* and*C. lusitaniae*. For the setS1 and S11 primers the PCR was performed in 25 µl reaction mixture consisting approximately of 5 µl of template DNA, 10 µl of Taq DNA Polymerase 2X ReadyMix, 1 µl of forward and reverse primers (1 µl) for each one and 2µl of PCR-grade water all of these were kept in a single tube. While for set S111 primers the amount of water was increased to the 4 µl in the reaction mixture. The species-specific primer pairs used in each mix and the calculated sizes of PCR products generated by each set of the mixes are listed in Table 1.

Table 1. Margin settings for A4 size paper

| Set No. | No | Target species | Forward primer | Reverse primer | Sizes of PCR product (bp) |
|---|---|---|---|---|---|
| Set S1 | 1 | *C. albicans* | CABF59 (5-TTGAACATCTCCAGTTTCAAAGGT-3) | CADBR125 (5-AGCTAAATTCATAGCAGAAAGC-3) | 665 |
| | 2 | *C. parapsilosis I* | CPPIF41 (5-TGACAATATGACAAAGGTTGGTA-3) | CPPIR122 (5-TGTCAAGATCAACGTACATTTAGT-3) | 837 |
| | 3 | *C. parapsilosis II* | CPPIIF41 (5-GGACAACATGACAAAAGTCGGCA-3) | CPPIIR69 (5-TTGTGGTGTAATTCTTGGGAG-3) | 310 |
| | 4 | *C. guilliermondii* | CGLF41 (5-CCCAAAATCACAAAGCTCAAGT-3) | CGLR61 (5-TACGACTTGAAGTTGCGAATTG-3) | 205 |
| Set S11 | 1 | *C. dubliniensis* | CDBF28 (5-AAATGGGTTTGGTGCCAAATTA-3) | CDBR110 (5-GTTGGCATTGGCAATAGCTCTA-3) | 816 |
| | 2 | *C. glabrata* | CGBF35 (5-CCCAAAAATGGCCGTAAGTATG-3) | CGBR103 (5-ATAGTCGCTACTAATATCACACC-3) | 674 |
| | 3 | *C. kefyr* | CKFF35 (5-CTTCCAAAGGTCAGAAGTATGTCC-3) | CKFR85 (5-CTTCAAACGGTCTGAAACCT-3) | 532 |
| | 4 | *C. krusei* | CKSF35 (5-GAGCCACGGTAAAGAATACACA-3) | CKSR57 (5-TTTAAAGTGACCCGGATACC-3) | 227 |
| Set 111 | 1 | *C. tropicalis I* | CTPIF36 (5-GTTGTACAAGCAGACATGGACTG-3) | CTPIR68 (5-CAAGGTGCCGTCTTCGGCTAAT-3) | 318 |
| | 2 | *C. tropicalis II* | CTPIIF36 (5-CTGGGAAATTATATAAGCAAGTT-3) | CTPIIR121 (5-TCAATGTACAATTATGACCGAGTT-3) | 860 |
| | 3 | *C. lusitaniae* | CLTF39 (5-CATGTCGAAATGCAACCCCCCG-3) | CLTR119 (5-GCGTACACTTGTGGCCATCTTTA-3) | 799 |

The PCR cycle parameters were as follows; one cycle of initial denaturation at 95 ˚C for 5 min; then 35 cycles of denaturation at 95˚C for 30 sec, annealing at 57˚C for 30 sec, and extension at 72˚Cfor 1 min; and followed by one cycle of final extension at 72˚Cfor 2 min. PCR products were analyzed using agarose gel electrophoresis in 1X TBE buffer at 100 V for 120 min in gel composed of 1.5% agarose gel. 100bp ladder DNA Marker was run with PCR products for sizing of the bands. Gels were stained with ethidium bromide solution (concentration of 0.5µg/ml) for 30 min, then visualized with a UV transilluminater and photographed.

## 3. RESULTS

Out of 283 urine samples examined (130 from males and 153 from females), 38(13.42%) were positive for *Candida* as shown in (Table 2).

Table 2. Distribution of *Candida* in urine samples among patients having urinary tract infections from both sexes and different ages cultured on Sabouraud's dextrose agar, incubation 48 hours at 37ºC.

| Gender | Age (years) | Number Samples Examined | No. Positive | Culture on Sabouraud's dextrose agar % |
|---|---|---|---|---|
| Male | 15-25. | 20 | 0 | (0/20) 0% |
| | 26-35. | 45 | 4 | (4/45) 8.88% |
| | 36-45. | 20 | 2 | (2/20) 10% |
| | >46 | 45 | 0 | (0/45) 0% |
| Total male | | (130/283) 45.93% | 6 | (6/130) 4.61 % |
| Total from all infection | | | | (6/38) 15.78 % |
| Female | 15-25. | 70 | 24 | (24/70) 34.28% |
| | 26-35. | 45 | 4 | (4/45) 8.88% |
| | 36-45. | 22 | 4 | (4/22) 18.18% |
| | >46 | 16 | 0 | (0/16) 0% |
| Total female | | (153/283) 54.06% | 32 | (32/153) 20.91% |
| Total from all infection | | | | (32/38) 84.21% |
| Total | | 283 | 38 | (38/283) 13.42% |

The percentages of positive samples among male patients were 4.61% while in females the positive samples with *Candida* were 20.91%. In male urine samples the highest percentage (10%) was among the age group 36-45 years followed by 8.88% in the age group 26-35 years, while in female urine samples, the highest positive percentage 34.28% was among the age group 15-25 years followed by 18.18% in the age group 36-45 years.

The isolates which were cultured on Sabouraud dextrose agar and showed positive results were re-cultured on Chromogenic Candida agar plate (Rapid Labs Ltd.,Essex, U.K.) and incubated at 37°C for 48 hr. Out of 38 samples 25 isolates were identified to their species level on Chromogenic Candida agar medium (Table 3).

These included *C. albicans* (7/38) 18.42%, *C. glabrata* (8/38) 21.05% and *C. Krusei* (10/38) 26.32%. Thirteen isolates were not diagnosed by Chromogenic medium, so 65.79% of the isolates were only identified.

Based on PCR results, five of primer pairs amplified species-specific DNA fragments from genomic DNA template, the size of PCR products was from 532 to 860 bp and they were of a specific size corresponding to each species of *Candida* (Figure 1 and 2).

Table 3. Identification of Candida species using Chromogenic Candida agar.

| *Candida* species | Culture on chromogenic agar |
|---|---|
| | Urine |
| *C. albicans* | (7/38) 18.42% |
| *C. dubliniensis* | ----- |
| *C. glabrata* | (8/38) 21.05% |
| *C. krusei* | (10/38) 26.32% |
| *C. tropicalis* | ----- |
| Not detected on Chromogenic agar | (13/38) 34.21% |
| *Candida* spp. identification | (25/38) 65.79% |

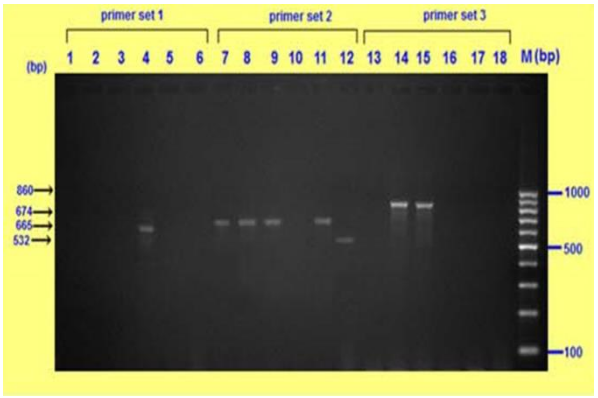


Figure 1. Agarose gel (1.5%) of PCR products obtained with species-specific primers. Lane (M) indicates 100-bp DNA marker.

Lane (1): *C. albicans*. Lane (2): *C. albicans* (665 bp) and lane (8): *C. krusei* (227 bp), this patient has dual Candida species infection by *C. albicans* plus *C. krusei*. Lane (9): *C. glabrata* (674 bp). Lane (10): *C. kefyr* (532 bp) and Lane (16): *C. tropicalis* II (860 bp), this patient has dual Candida species infection by *C. glabrata* plus *C. tropicalis II*. Lane (12): *C. kefyr* (532 bp).

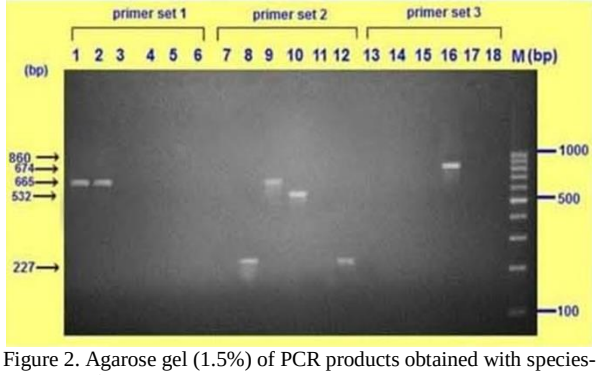


Figure 2. Agarose gel (1.5%) of PCR products obtained with species-specific primers. Lane (M) indicates 100-bp DNA marker.

Lane (4): *C. albicans* (665 bp). Lane (7 and 11): *C. glabrata* (674 bp). Lane (8): *C. glabrata* (674 bp) and lane (14*): C. tropicalis* II (860 bp), this patient has dual *Candida* species infection by *C. glabrata* plus *C. tropicalis II*. Lane (9): *C. glabrata* (674 bp) and lane (15): *C. tropicalis* II (860 bp), this patient has dual Candida species infection by *C. glabrata* plus *C. tropicalis II*. Lanes (12): *C. kefyr* (532 bp).

infection by *C. kefyr* plus *C. tropicalis II*. The sample in lanes (5, 11, and 17): did not show any result. Lane (12): *C. krusei* (227 bp).

The DNA of *C. albicans*, *C. tropicalis II*, *C. glabrata*, *C. kefyr* and *C. Krusei* was amplified by PCR and generated DNA fragments of 665, 860, 674,532 and 227 bp respectively (Figure 1 and 2). These were the exact sizes corresponding to each species.

Among urine samples, 86.85% contained only one species of *Candida* and the rest 13.15% contained more than one species of *Candida* (Table 4). The prevalence of different species of Candida was as follow: The species isolated most frequently from urine samples were *C. Krusei* (26.3%) followed by *C. kefyr* (23.68%), *C. glabrata* (18.42%), *C. albicans* (15.78%), *C. glabrata + C. tropicalis II* (7.89%), *C. albicans + C. krusei* (2.63%) and *C. kefyr + C. tropicalis II* (2.63%).

Table 4. Distribution and identification of *Candida* species using Multiplex PCR.

| *Candida* species | PCR-based identification |
|---|---|
| | Urine |
| *C. albicans* | (6/38) 15.78% |
| *C. albicans + C. krusei* | (1/38) 2.63% |
| *C. albicans + C. tropicalis II* | ----- |
| *C. dubliniensis* | ----- |
| *C. glabrata* | (7/38) 18.42% |
| *C. glabrata + C. tropicalis II* | (3/38) 7.89% |
| *C. krusei* | (10/38)26.31% |
| *C. tropicalis II* | ----- |
| *C. kefyr* | (9/38) 23.68% |
| *C. kefyr + C. tropicalis II* | (1/38) 2.63% |
| *C. guilliermondii* | ----- |
| Not identification | (1/38)2.63 % |
| PCR-based foridentification *Candida* species | (37/38)97.37% |
| Percentage of samples contained only one species of Candida. | 86.85% |
| Percentage of samples contained more than one species of Candida. | 13.15% |

## 4. DISCUSSION

The result of this study showed that *C. krusei* was more common in urine samples with a prevalence of 26.31% followed by *C. kefyr* (23.68%), *C. glabrata* (18.42%), and *C. albicans* (15.78%). This finding is not in agreement with other studies conducted in Turkey by Ece (2014) who revealed that *C. albicans* is the most common isolated species with prevalence of 56% and in Iran by Mahmoudabadi and his collogues (2002) who revealed that *C. albicans* was the most common species among the isolates (93.9%) followed by *C. glabrata* (2%), *C.dubliniensis* (2%), and *Candida* species (2%). Also, the present study did not agree with the studies of Ozahak-Baysan *et al.*, (2012) who used a similar PCR technique to detect *Candida* species isolated from urine of 100 patients. The most common species they recovered were *C. albicans* with a prevalence of 44% of all yeasts followed by *C. tropicalis* (20%) and *C. glabrata* (18%). However, Jaya and Harita (2013) from India reported prevalence rates of *C. tropicalis* (60.76%) followed by *C.albicans* (26.71%), whereas, *C.glabrata* (35.15%) was reported by Adjapong *et al.* (2014) as the most prevalent species in urine samples from patients in Ghana, followed by *C.albicans* (27.7%) and *C. tropicalis* (20.5%).

*Candidiasis* is a common worldwide infection with the most common *species being* is *C. albicans* in USA and Northern and Middle Europe, while non-*albicans* strains are common in Asia, South Europe, and South America. On the other hand *C. glabrata* was commonly isolated in the USA and North and Central Europe from cases of candidemia (Falagas *et al.*, 2010) this was confirmed also in the present study.

## 5. CONCLUSION

The results of this study showed that *C. Krusei* is the most frequently isolated species from urine samples with a prevalence of 26.3% followed by *C.kefyr* (23.68%), *C.glabrata* (18.42%) and *C. albicans* (15.78%). Phenotypic assay based on the chromogenic Candida agar was preliminary presumptive test, but is not accurate and precise for the identification of *Candida* species.

## 6. ACKNOWLEDGEMENTS

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**کورتیا لێکولینێ:**

پشکنین و دیارکردن داده برێت پزیشکی یه جۆریت کاندیدا به سیسته می کلاسیک و گه ردیله یی ته کنیکی به کارهێنان جۆر دیاریکراو تێکه ڵ یه په ڕتوکی خوێندنه وه (Multiplex PCR)

نفشێت کاندیدا Candida یێت که ڕووویان ژزیندیکێت سروشتی یێت کو د ناف چاریێت کلمیشکی دژین، نه خاسمه بووڕیکێت ژوور یێت هه ناسێ و کاریژکێت ئویرك ورویقیکا و ئاویزی. چه ندنفشێت کاندیدا دبنه ئه گه ڕا هنگافتنێت که ڕووییێت ده لیقباز بو وان که سێت بێچاره ژ لایێ به رگرین ڤه. 283 مه سته ریێت میزی. و هه می مه سته ڕه هاتنه هه لنێران بو هه بوونا نفشێت کاندیدا ب کارئینانا سابووراود ده کستروز ئاگار Sabourauds dextrose agar. ب کارئینانا ناڤه ندا کروموجینیك کاندیدا ئاگار chromogenic candida agar ده ستنیشانکرنا مه گرتی یا نفشێت کاندیدا سێ نفش دیارکرن، ئه وژی C. albicans, C. glabrata, C. krusei, نفشێت C. krusei ژ ژێده ریێت مه سته ریێت هه مه جوور هاتنه ڤه دیتن.لپه ی ڕێبازا Multiplex PCR، بینج نفشێت کاندیدا هاتنه ده ستنیشانکرن، ئه وژی:C.tropicalis II, C. albicans, C. glabrata, C. kefyr, C. krusei نفشێ C. kefyr ژ مه سته ره کا (سه ڕپه مبیکێ) ئاویزی هاتنه ده ستنیشانکرن، لپه ی ڕێبازا Multiplex PCR

**خلاصة البحث:**

هدفت الدراسة الحالية الى عزل وتشخيص انواع خميرة ال *Candida* من عينات ادرار مرضى يعانون من التهابات في الجهاز البولي في محافظة دهوك. شخصت الانواع اعتمادا على انواع مستعمراتها الظاهرة على وسط Chromogenic Candida agar فضلا على استخدام تقنية Multiplex PCR . اظهرت النتائج سيادة انواع Non- albicans Candida.. حيث عزلت الC.krusei باعلى نسبة (26.3%) تلتها C.kefyr بنسبة (23.68%) ومن ثم C. glabrata بنسبة) 18.42%( . اما C. albicans ظهرت بنسبة ( 15.78%) . استخدام الطرق الجزيئية اعطت نتائج اكثر دقة مقارنة باستخدام الطرق الزراعية المظهرية.