**Journal for Research in Applied Sciences and Biotechnology**

www.jrasb.com

**ISSN: 2583-4053**

Volume-2 Issue-6 || December 2023 || PP. 137-144

https://doi.org/10.55544/jrasb.2.6.20

# Molecular Detection and Identification of *Candida* Species Isolates from Oral by RFLP-PCR

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**www.jrasb.com || Vol. 2 No. 6 (2023): December Issue**

| **Received:** 16-12-2023 | **Revised:** 23-12-2023 | **Accepted:** 28-12-2023 |
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**ABSTRACT**

**In this study, 10 local isolates from a total of 50 samples of *Candida* sp. were collected from oral swabs of patients with oral infections in Mosul hospitals. The isolates were diagnosed based on culturing, microscopic and biochemical characteristics, and then molecular methods. The first diagnosis by culturing, microscopic and biochemical tests found the isolates were identified as *Candida* sp. The ITS region was amplified using universal primers (ITS4-ITS5), The PCR product was size (510-721) bp. Performing RFLP-PCR using M*sp*I, H*ha*I,and E*co*RI, restriction enzyme to detect and identify *Candida* species, the results showed the presence of the cutting sequence of M*sp*I and H*ha*I enzymes in the genomic DNA content of local isolate and the absence of the sequences for the E*co*RI restriction enzyme. Two *Candida* species were identified *(C. krusei and C. kefyr* the basis of size and fragment sequences then compared with sequences of standard strains from the gene bank in previous studies. Therefore, it can be observed that there is a genetic variation between the local isolates and that there are different genotypes of rDNA 5.8S have been diagnosed in 10 isolates after the cutting process with three restriction enzymes. We conclude from this study that the RFLP-PCR technique was the best in diagnosing and identifying *Candida* species compared with traditional methods. and we are d the genetic variation between local isolates.**

***Keywords-*** *Candida*, Genetic variation, Restriction enzyme, RFLP-PCR.

## I. INTRODUCTION

*Candida* is one of an opportunistic pathogen that can cause disease and transmission of a weakened immune system such as in patients with COVID-19, HIV due to chemotherapy for cancer patients or after wrong treatment with antibiotics [1]. *The Candida* genus includes approximately 150 species or more that show different levels of resistance to antifungal agents. Thus, it is important to diagnose the organism that causes the disease certain *Candida* species such as *C. dubliniensis* which in particular is very difficult because of its similarity with *C. albicans* [2]. The genus of *Candida* is That is Widespread and has many factors of virulence, in addition to ability to injury to the respiratory, digestive, urinary and genital tract [3,4]. In some cases, it has entered the bloodstream [5]. The infection caused by *Candida* is a heavy burden on Public health, due to the high mortality rate [6]. The *C. glabrata* It is one of the most important types of *Candida* in terms of its pathogenicity After *C. albicans*, *C. glabrata.* is characterized by its inability to form fungal pseudo forms. It does not have the ability to dimorphic transform, but it forms blastopore, and its colonies are characterized by being smooth, soft and creamy white in color [7]. Timmerman and others showed that yeast *C. glabrata* is a fungal pathogen that threatens the lives of patients and

**ISSN: 2583-4053**
Volume-2 Issue-6 || December 2023 || PP. 137-144
https://doi.org/10.55544/jrasb.2.6.20

causes more problems in hospitals because it shows self-resistance to antifungal drugs [8,9].

Many appearance features can be used to identify isolated yeasts, which may take days or weeks for developing higher on the culture [1]. Molecular techniques that use DNA amplification are an alternative method of diagnosing and identifying some organisms. Candida types have been identified by RFLP-PCR of the rDNA [10]. In one study conducted in China on three species of fungi (*Candida*, *Aspergillus*, and *Cryptococcus*) by the RFLP-PCR technique where regions (ITS1 and ITS2) were amplified in rDNA 5.8S to detect DNA of fungi [11]. Thus, they documented that this technique is sensitive, specific, and fast, and they were used to diagnose fungal infections in immune-compromised people. The RFLP-PCR technique is one of the most important and best techniques used in the diagnosis of yeasts, especially the pathogenic and isolated Candida from infected people. This technique has been used extensively in diagnostic processes and the identification of pathogenic organisms [12]. The aim of the study is to detect and diagnose the pathogenic Candida species by morphologic, microscopic, biochemical test and molecular methods. To find the genetic variation between Candida isolates through the cut sites where the restriction enzymes cut off by RFLP-PCR technology.

## II. METHODOLOGY

### *2.1 Yeast Isolate*

Ten yeast isolates of *Candida* from a total of 50 samples were taken from oral swabs of people suffering from oral infections of different age groups and both sexes in the Al-Salam Teaching Hospital between July onto September 2021. The samples were placed in sterile distilled water for 10 minutes, and then a series of dilutions were prepared 0.1 ml of each dilution was placed on (Yeast Malt Extract) medium containing 50 mg of antibiotic (Chloramphenicol) and incubated at 37ºC ± 1 for 24 hours. Yeast isolates were cultured on Sabouraud agar to develop the yeast isolates and keep them in the refrigerator until use. The media were prepared according to the supplied company instructions

*Preparation of media***:**

**A- Yeast Malt Extract Agar Medium(YMA):** This medium is used to isolate samples composition of (g.l): 3 g of yeast extract, 3 g of malt extract, 5 g of peptone, and 15 g of agar, dissolved in 1 L of D.W. and the pH was adjusted at =7.0 (13).

**B- Malt Extract Agar medium(MA):** prepared 1 liter of this medium: 20 g of Malt extract, 1 g of peptone, 20g of glucose, 20g of agar was dissolved in sterilized D.W. and pH was adjusted at = 5.6 [13].

### *2.2 Biochemical tests:*

**1- Catalase Test:** Yeast colony of (48-h) growing was taken on glass slides and one drop of $H_2O_2$ (3%) was added. The appearance of a gas bubble indicated the presence of a catalase enzyme [14].

**2- Gelatin Melting Test:** This test was used to find out the ability of yeasts to produce the analyzed enzyme by pollinating test tubes containing a gelatin medium with a portion of the colony using an acupuncture method after incubating at 37ºC for 48h. Then the tubes were placed in the refrigerator at 4ºC.for later examination [15].

**3- Sugar fermentation test:** This test was performed by taking a lube campaign from each sample and placed in the fermentation medium composed of (g.l): 4.5 g of yeast extract, 7.5 g of peptone 40 ml of phenol red. The volume was completed with distilled water and set the pH at = 5.6, As the medium turns dark red, then sterilizes with the sterile device, and after it cools, three types of sugars at a concentration of 2% each separately (glucose- lactose- mannose- galactose- maltose- sucrose- xylose- arabinose) was added [16].

**4- Germ tube formation:** In a test tube 0.5 ml of Sabouraud Dextrose broth medium was placed, and colonies were added to it, incubated at a temperature of 37 °C for (2-3 h), after that a drop of the suspension was placed on a clean glass slide with a cover placed on it and examined under the light microscope to find the germ tube. [17].

**5- Chromogenic Agar test:** *Candida* isolates from Sabouraud Dextrose Agar medium were transferred to HiCrome *Candida* differential agar medium and incubated at 37 °C for 24 h. The identified *Candida* isolates types by colony color as according to the manufacturer's instructions. [18].

### *2.3 Molecular Analysis*

**1- DNA Extraction:** DNA was extracted from *Candida* yeast local isolates by (Fungi / Yeast GDNA Extraction Mini Kite) provided by Korean company Favorgen, following the protocol steps of the Kite. The DNA purity and concentration were determined using nanodrops.

**2- Specific PCR:** PCR for yeast identification was performed using two universal primers ITS4 Forward (5`TCCTCCGCTTATTGATATGC3`) andITS5Reverse(5`GGAAGTAAAAGTGCTAACAAG G3`) [19]. The amplification reaction was performed by Eppendorf. each reaction mixture contained 12.5 µl (PCR Master Mix 2x), 1 µl with concentration (10 pmol. µl) from each primer, (4 µl) with concentration (100 ng) DNA (measured by NanoDrop.), the volume completed to (25 µl) by sterile Distilled Water. The tubes are placed in Eppendorf® Mastercycler within the following program: initial denaturation at 95°C for (5 min), followed by 35 cycles of Denaturation at 95 ° C for (45 s), Annealing at 57°C for (1 min), Extension at 72°C for (1 min), and last elongation stage of 72ºC. for (7 min). Final incubation at 4 ºC. After the program was ended, The PCR products were loaded in 2% agarose gel and the gel was running (45 min) at100 V, then illuminated by UV

**ISSN: 2583-4053**
Volume-2 Issue-6 || December 2023 || PP. 137-144
https://doi.org/10.55544/jrasb.2.6.20

Transilluminator at 320 nm and photographed by digital camera.

**3- RFLP-PCR:** Amplification products were digested with three restriction enzymes (E*co*RI, M*sp*I and H*ha*I) separately to identify *Candida* species and to find the genetic variation in the rDNA cut sites by using sterile Eppendorf contains (25 µl) of the PCR product, (0.5µl) of the restriction enzyme and the final volume completed to (25.5µl), the contents are mixed and placed in the centrifuge for several seconds and incubated at 37º C the optimum temperature of the enzyme, followed by loading in agarose gel at 2%.

***2.4 Statistical Analysis:***

The computational program SPSS version 25 were used in data analysis to test for significant differences when compare the frequency of detection for each species in addition to compare detection by RFLP-PCR technique with traditional methods, A P value < 0.05 was considered statistically significant.

**1- Isolation and diagnosis:** The results revealed that all local isolates belong to *Candida's* yeast according to the morphological, cultural, microscopic feature, biochemical tests, and molecular method as a follow:

**2- Colony and Cultural Morphology:** The grown colonies on the (YMA) medium showed similar morphology, white to creamy in color circular form, and smooth. (**Fig.1**). These characteristics obtained by the authors are similar to the characteristics of the *Candida* colonies when grown on the SDA medium. These results agree with many reports in terms of the appearance of creamy colonies, smooth and rounded gloss to provide appropriate culture conditions [20,21].

**Figure 1: local isolates of *Candida* sp. On (YMA) medium.**

**3- Microscopic Characteristics:** The yeast isolates gave positive results for the Gram dye and the cells vary shape from oval to spherical, elongated, or cylindrical (**Fig. 2**), This result was in accordance with researchers [22], the emergence of *Candida* cells blue-dyed because retaining of the Peptidoglycan layer in the cell wall of this pigments.

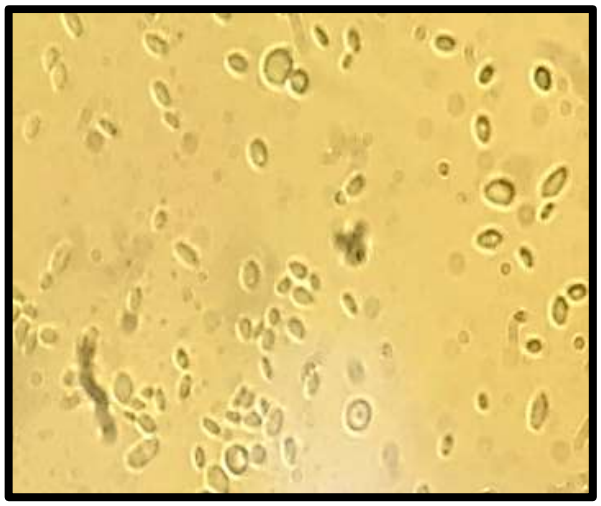

**Figure 2: *Candida* cells under the microscope (100 X)**

***Germ Tube Formation:***

The results detected that all local isolates were can not form germ tube after incubation period at 37º C for (2-3 h) in (0.5 ml) of serum. This result is corresponding with many researchers who mentioned that only *C. albicans* can form germ tubes [23,24].

**3- Chromogenic agar test:** The results of growth on chromogenic agar medium showed the colonies in different colors, which are pink and purple (**Fig. 3**). Each color is considered a diagnostic characteristic for a certain type of Candida species, because it contains a chromogenic mix, which is a substrate based on which the enzymes of each Type. The principle of work of this medium depends on its containing of basic color-generating substances called chromogenic, which determines the species by secreting yeast species of their enzymes that give special colors when interacting with the medium. Each type is dyed in a specific color. These results agree with [18,25] who reported that the colors of *Candida* sp. Are different according to the species and each color is a diagnostic characteristic of that type, and it was identical to the colors of the manufacturer of the medium.

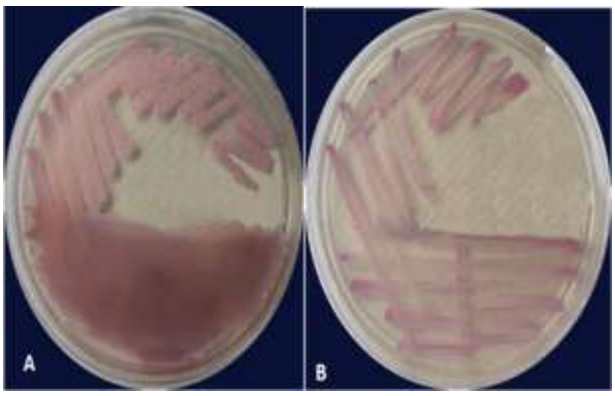


**Figure 3: Results of isolates growth on Chromogenic agar medium: A-*C. krusei*, B- *C. kefyr***

**Journal for Research in Applied Sciences and Biotechnology**

www.jrasb.com

**ISSN: 2583-4053**

Volume-2 Issue-6 || December 2023 || PP. 137-144

https://doi.org/10.55544/jrasb.2.6.20

## III. RESULT

***3.1 Biochemical tests:***

**1-Sugars Fermentation Test:**

All local isolates showed able to ferment the sugars (glucose, maltose, galactose, lactose) through conversion color of the medium from red to yellow. Meanwhile, all isolates weren't able to ferment the sucrose except (YS7, YS9) isolates that appear to ferment all sugars except lactose (**Table 1**). These results are consistent with some reported showed that all types of Candida spp. were able to ferment sugars [22] and agree with others which showed that all types of *Candida albicans* and *C.tropicales* were able to ferment glucose but not able to ferment sucrose [26].

**Table 1: Fermentation reactions of isolated yeast.**

| Isolate | Sucrose | Glucose | Maltose | Galactose | Lactose | Arabinose | Mannose | Xylose |
|---|---|---|---|---|---|---|---|---|
| YS1 | - | + | + | + | + | + | + | + |
| YS2 | - | + | + | + | + | + | + | + |
| YS3 | - | + | + | + | + | + | + | + |
| YS4 | - | + | + | + | + | + | + | + |
| YS5 | - | + | + | + | + | + | + | + |
| YS6 | - | + | + | + | + | + | + | + |
| YS7 | + | + | + | + | - | + | + | + |
| YS8 | - | + | + | + | + | + | + | + |
| YS9 | + | + | + | + | - | + | + | + |
| YS10 | - | + | + | + | + | + | + | + |

**(+) refers to fermentation ability. (-) no fermentation occurs.**

**2-Catalase test:** The results indicate that all isolates were positively responded to catalase enzyme production analysis. These results agree with [21] which mentioned that all yeast isolates have the ability for catalase enzyme production same.

**3-Gelatin Liquefaction test:** The results indicate that all isolates were couldn't gelatin liquefaction, because were unable to production gelatinase enzyme. These results were corresponded with [27] who reports that all yeast isolate is unable to gelatin liquefaction**.**

***3.2 Molecular Diagnosis:***

**1- DNA Extraction:** After extraction of the DNA, the concentration and purity of the DNA was measured with a nanodrop device. The concentration within the normal range between (1.6 - 1.8). This result is consistent with many reports. [12,27].

**2- Specific PCR:** From **(Fig.4**), the results reveal the presence of 7 bands of the same size (510) bp. which was the isolates (YS1, YS2, YS3,YS4,YS8, YS9,YS10),while the isolates (YS5,YS6,YS7) reveal 3 bands of the size (721)bp. Thus, the presence of these brands of the same size indicates that this isolates belong to the same type, and indicate the local isolates belong to the two species of candida. The universal primer (ITS4, ITS5) were able to amplified the ITS region of local isolates, the PCR product size approximately (510,721)bp. These results are consistent with research results [28]. Who were a bled to successfully amplified the ITS region of all yeasts tested providing PCR product of approximately (510-870) bp. and the isolate C. *krusei* was size (510) bp. When using universal primers (ITS1-ITS4).

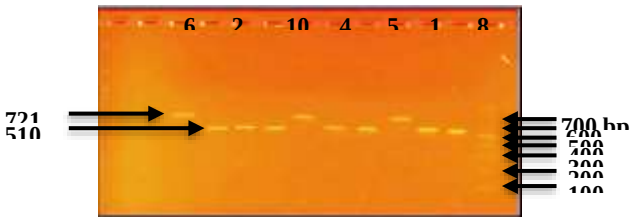


**Figure 4: The PCR reaction products of *Candida sp.* yeast of (510-721) bp, which was loaded at 2%. M:**

***3.3 Marker. (1-10): Sample***

**1- RFLP-PCR:** The results of cutting using the M*sp*I restriction enzyme showed that the local isolates (1,2,3,4 ,8,9,10) (**Fig. 5**) had two bands with a size of (190,320) bp as *C. krusei*, while samples No. (5,6,7) showed had three bands with different size (320,210,190) bp as *C. kefyr* when were loaded in the agarose gel at 2%. These sizes are consistent with the expected gene sizes after comparing the digested part size with reference bands for species identification [28,29] (**Table 2**).

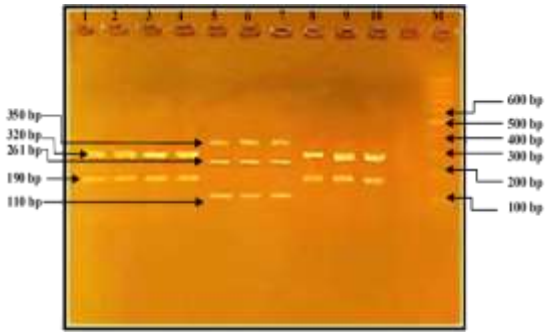


**Figure 5: The results of the PCR reaction of the *Candida* sp. IST4 gene after restriction digestion with the enzyme M*sp*I. M represents Marker. The samples(1,2,3,4,8,9,10) *Candida krusei* and the samples (5,6,7) *Candida kefyr.***

The presence of two or three bands of DNA resulting from the PCR and digestion with the *MspI* restriction enzyme indicates the availability of nitrogenous bases sequences significantly for the M*sp*I enzyme in the genomic content of the local isolates and at which can cut these fragments. The results of cutting using the *HhaI* enzyme also showed that isolates (1,2,3,4,8,9,10) had two bands with sizes (190,320,) bp as *C. krusei* (**Figure 6**), while the samples (5,6,7) had three bands with sizes (190,211,320) bp as *C. kefyr* When they were loaded in the agarose gel at 2% (Table 3). On the other hand, when digestion the PCR product of local

**Journal for Research in Applied Sciences and Biotechnology**

www.jrasb.com

**ISSN: 2583-4053**

Volume-2 Issue-6 || December 2023 || PP. 137-144

https://doi.org/10.55544/jrasb.2.6.20

isolates with E*co*RI enzyme, it was found that this enzyme did not recognize its cutting region in the genomic DNA bands when they were loaded.

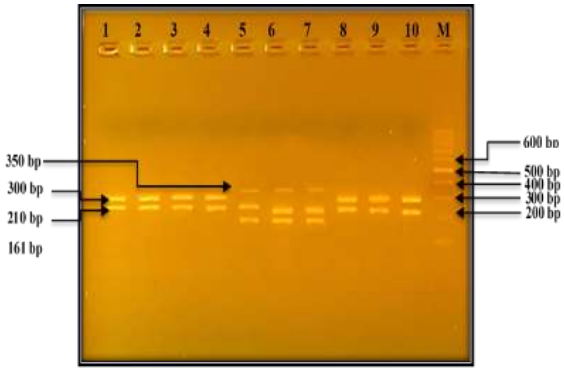


**Figure 6: The results of the PCR reaction of the *Candida* sp. IST4 gene after restriction digestion with the H*ha*I, as M, represents Marker. The samples (1,2,3,4,8,9,10) *Candida krusei* and the samples (5,6,7) *Candida kefyr*.**

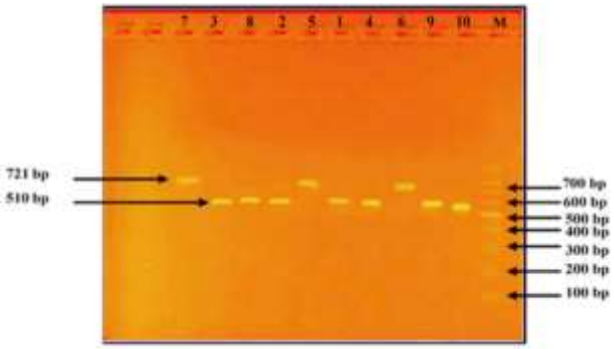


**Figure 7: The result of the PCR reaction of the *Candida* sp. genus ITS4 after restriction digestion with the enzyme E*co*RI. as M, represents Marker.**

**Table 2: Sizes of ITS4-ITS5 PCR products for *Candida* species before and after digestion with M*sp*I**

| *Candida* species | Size of ITS4, ITS5 | Size of restriction products | Number | % |
|---|---|---|---|---|
| *Candida Krusei* | 510 | 320,190 | 7 | 70 |
| *Candida kefyr* | 721 | 110,261, 350 | 3 | 30 |

**Table 3: Sizes of ITS4-ITS5 PCR products for *Candida* species before and after digestion with H*ha*I**

| *Candida species* | Size of ITS4, ITS5 | Size of restriction products | Number | % |
|---|---|---|---|---|
| *Candida Krusei* | *510* | *210,300* | *7* | *70* |
| *Candida kefyr* | *721* | *161,210 ,350* | *3* | *30* |

## IV. DISCUSSION

The results of the RFLP-PCR technique which used in this study are agreement with [28] Whose used RFLP-PCR technology to diagnose six medically important isolates of the Candida sp. and digested them with the restriction enzyme *MspI* and compared them with 8 standard strains from the gene bank based on the size of the PCR product and the size of the fragments digested with the restriction enzyme. also agree with [29] who isolated 98 isolates candida from urine clinical samples and identified using PCR-RFLP by *MspI*, the size of the PCR product was (383-871) bp, most of the *Candida* isolates were *Candida albicans* (535bp) in 41 cases, *Candida glabrata* (871bp) in 16 case, *Candida tropicalis* (522bp) in 12 cases, *Candida krusei* (510bp) in 10 cases, *Candida parapsilosis*(520bp) in 8 cases and *Candida kefyr* (721bp) in 2 cases. In other study, [12] when using RFLP-PCR technique to diagnose 30 isolates of *Candida* sp. by M*sp*I restriction enzyme, and to find a genetic variation between local isolates. also that the restriction enzyme sequences were found in the genomic DNA content of the local isolates. And consistent with [27] who used RFLP-PCR to identify 80 clinical isolates and three standard strains by *MspI* and *BlnI* enzymes separately, the size of the PCR product and *MspI* digestion were similar for each *Candida* species identified by the standard strain. In this study, the results were consistent with [30], who identified 149 types of Candida isolated from patients' vaginas by RFLP - PCR technique and using the enzyme M*sp*I. was 89% of the isolates belonged to *C. albicans* followed by *C.galabrata* (5.4%) and C. *kefyr* (1.4%). And agrees with [31] Which diagnosed 73 urine samples using RFLP-PCR technology by *MspI* enzyme, the results after sending the samples to determine the sequence of nitrogenous bases showed that the samples were as follows: *C.albicanis, C.krusei, C.glabrata, C.tropicalis.*

These results indicate that there is *MspI* and H*ha*I enzyme cutting sequences in the genomic DNA content of local isolates and the lack of sequences to restriction enzyme E*co*RI. Therefore, this results may indicate there is a genetic variation between the local isolates and that there are different genotypes of rDNA 5.8S have been diagnosed in 10 isolates after the cutting process with three restriction enzymes and that this technique assists in the diagnosis of local isolates depending on the cutting sites and the nitrogenous bases sequences that have been cut with the restriction enzymes. Also the results indicate that the RFLP-PCR technique has a very high discriminating power in identifying and diagnosing Candida species compared to traditional methods. These results concurrence with [27] When our used *MspI,BlnI* enzyme in the REFLP-PCR technology, so the results showed that both enzymes succeeded in finding the cutting regions and that the enzyme sequences were found in the genomic DNA content of the local isolates. As shown, the B*ln*I enzyme was better at finding the genetic variation between local isolates and

**ISSN: 2583-4053**
Volume-2 Issue-6 || December 2023 || PP. 137-144
https://doi.org/10.55544/jrasb.2.6.20

diagnosing isolates according to similar or different cutting regions. And agrees with [18] reported that 194 samples were diagnosed using Chromogenic Agar and RFLP-PCR by M*sp*I enzyme, the results showed the diagnosis of the isolates as follows: *C.albicanis* (, *C.krusei, C.glabrata, C.tropicalis*, and they were mentioned RFLP-PCR method has a better diagnostic and discriminatory ability to identify Candida species than Chromogenic agar [18]. In other study, were identified 58 *Candida* sp. strains isolated from animals using (PCR-RFLP) technique using M*sp*I, B*siS*I restrictions enzyme which appeared similar specificity for the identification of *Candida* strains, RFLP- PCR represents a reliable, quick and relatively inexpensive genotyping method, recommended for rapid identification of *Candida* sp. [32]. The early diagnosis of invasive fungal infections is necessary for decrease of infections rate. [28].

In the present study the results showed that the culture, microscopic, biochemical tests, even the germ tube test and chromogenic agar are unreliable results compared with RFLP-PCR method, because these methods does not identify *Candida* at the level of genus and species, it was only determines whether these isolates are Candida or other yeasts, while the RFLP-PCR method determined at the species level, may be the reason is that similar enzymes can be produced by different types of *Candida* or that the reaction of the enzyme-substrate was not alone in all types of *Candida*, then a similar color was produced by more than one species and therefore the chromogenic medium was not able to diagnose the species as mentioned Manufacturer's instructions [18]. The time taken for RFLP-PCR is similar to traditional methods, but the RFLP method is excellent in identifying all types of Candida. The sensitivity of RFLP-PCR was100% compared to traditional methods, so it is more reliable for distinguishing between *Candida* types than traditional methods, although it may be preferred in many other researches and studies in determining the types of *candida*. Genotyping methods are more accurate and sensitive in identifying *Candida*. DNA amplification by universal primers then detection using cut regions and sequences which can that have been cut with the restriction enzymes, enhances sensitivity in identification of *Candida* significantly. Molecular techniques are good substitute for identification and diagnosis of fungus such as *Candida sp.*, because of rapid, easy and It has a high discriminatory and diagnostic power. This procedure was used in species diagnosis especially in the epidemiological studies to choose appropriate antifungal drugs [33].

The ITS region is important in identifying *Candida* species as it contains several highly conserved sequences, but there is variation in sequences in other regions of this molecule, which serves as a marker for restriction length polymorphisms. This region has been used by many researchers to identify *Candida* species and some medically important fungi using different methods such as DNA probes, Nested PCR, Sequencing, RFLP-PCR [28]. The ITS4, ITS5 region is surrounded by a gene 5.8 rDNA is suitable for the diagnosis, identification and classification of fungi and is important from a medical point of view because it helps in the early detection of diseases and in the curative. The ITS region of rDNA which were Amplificated and digested using restriction enzymes enables the identification of Candida species based on size and sequence change [27].

## V. CONCLUSION

We conclude from our current study that there is M*sp*I and H*ha*I enzyme cutting sequences in the genomic DNA content of local isolates and the lack of sequences to restriction enzyme *EcoRI*. Therefore, may there is a genetic variation between the local isolates and that there are different genotypes of rDNA 5.8S have been diagnosed in 10 isolates after the cutting process with three restriction enzymes and that this technique assists in the diagnosis of local isolates depending on the cutting sites and the nitrogenous bases sequences that have been cut with the restriction enzymes. RFLP-PCR technology is the best in identifying *Candida* species than traditional methods.

## ACKNOWLEDGMENTS

This work was carried out by the Department of biology/ College of Education and Pure Sciences University of Mosul / Iraq and with the support of the Genomic Center in the DNA lab/ Mosul/Iraq.

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**ISSN: 2583-4053**
Volume-2 Issue-6 || December 2023 || PP. 137-144
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