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Molecular Identification of Medically Important Candida Species Isolated from Pregnant Women Using PCR-RFLP

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Abstract: *Background/Aim*: *Candida* species; an opportunistic eukaryote, is considered one of the most prevalent pathogens causing serious illness in immunocompromised patients. It is not uncommon for every woman to catch vaginal candidiasis once or more in her life. the majority of *candida* infection in human is represented by five species namely *Candida albicans, Candida glabrata, Candida tropicalis, Candida parapsilosis and Candida krusei*. The present study aims to evaluate rapid, accurate and reliable identification of candida organisms to the species level. *Materials and Methods*: A total of 138 *Candida* species isolated from urine samples of some pregnant women were subjected to traditional and DNA-based analysis using phenotyping and PCR-RFLP respectively. PCR amplification was performed to 5.8s region of rDNA then the amplicons were digested separately by Msp I and Hae III restriction enzymes and the products were displayed by Agarose gel electrophoresis. *Results*: 82 out of 138 isolates were identified as *Candida albicans*, with a percentage of 59.0, *Candida glabrata* was represented by 26 isolates (19.0%). 14 isolates exhibited the characters of *Candida tropicalis* with a percentage of 10, 10 isolates showed the characters of *Candida krusei* (7.0%) and 8 isolates appeared to be *Candida parapsilosis* by (5.0%). *Conclusion*: PCR-RFLP appeared to be a very accurate, rapid and reliable assay for the identification and differentiation of *candida* species.

Key words: Molecular Identification · Candida Species · PCR-RFLP · Restriction Enzymes · Msp I - Hae III

INTRODUCTION

Candida species are the yeast that belong to kingdom Fungi. *Candida* are eukaryotic microorganisms, consist of 1, 500species by percentage 1 only of all yeast species. But more than 200 of the Candida species is identified, most of which exist as saprophytes organisms. *Candida albicans* is the most general species, followed by further pathogenic species which include *C. glabrata, C. parapsilosis, C. krusei, C. tropicalies* and *C. kefyr* [1].

Candida species is the most common cause of invasive fungal infections [2] *Candida species* ranks as the fourth most common cause of nosocomial bloodstream infections and the mortality of these infections does vary between 33% and 75%. The risk of infection is increased opportunistic in the immunocompromised including diabetic, cancer patient and pregnant women [3].

Candida species causes Vulvovaginal candidiasis (VVC), Which is a common disorder in women [4]. The majority of cases of VVC are caused by *Candida albicans*; however, episodes due to non-albicans species of *Candida* appear to be increasing in immunocompromised as well as pregnant women [5].

The risk of infection is especially high in the immunocompromised, hospitalized patient. The treatment of and prophylaxis for Candida infection have led to the emergence of resistant species and the acquisition of resistance in previously susceptible species. Current therapeutic options include amphotericin B and its lipid compounds, fluconazole, itraconazole, voriconazole and caspofungin. Azoles are the drug of choice for VVC; however, resistance has been reported especially in *non-albicans Candida* species [6]. Because of the different susceptibility of *Candida species* to antifungal agents, it is important to definitely identify the causative *Candida* to the species level [7]. Moreover, the conventional methods are time-consuming and may lead to misdiagnosis among closely related species. Therefore, molecular methods may provide a rapid and accurate alternative [8].

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So, the aim of this study was to prove the rapidity, reliability and accuracy of PCR-RFLP for the identification of medically important *candida* species against conventional identification.

MATERIALS AND METHODS

Isolation and Purification: Urine samples from some pregnant Egyptian women were collected from march/2019 to February/2020. Samples were collected from private laboratories for medical microbiology analysis-Cairo, Egypt and sheets of consent approval and questionnaire were taken from the under-trial women.

Urine samples were firstly centrifuged and examined microscopically then the suspected yeast containing sediments were separately inoculated into tubes containing Sabouraud dextrose broth (SDB). The inoculated tubes were incubated at 30°C for 28-36 hours [9]. A loopful was taken from each turbid SDB tube and streaked onto Sabouraud dextrose agar (SDA) plate, then SDA plates were incubated for 24-48 hours at 30°C [10].

Identification: One single colony grown on each SDA plate was picked up then streaked onto CHROMagar candida plate [11]. And the plates were incubated at 30°C for 24-48 hours. After incubation the color of grown colonies on each plate was observed and recorded.

A germ tube test was performed on green colonies that appeared on the CHROMagar plate. One single green colony was taken from a CHROMagar plate then inoculated in an Eppendorf containing 300 µl fresh human serum and incubated at 37°C for3 hours. One drop from the incubated Eppendorf was poured on a slide and examined microscopically using oil immersion lens to observe the presence of germ tube as elongated stalk protruded from mother cell [12].

Biochemical identification of the isolated yeast was performed using API 20C. A pure single colony obtained from SDA plate was immersed into API 20 C suspension tube and the turbidity was visually adjusted with McFarland tube 2.0. The wells API 20C strip were inoculated with the suspension and the strip was incubated for 24 -48 hours at 30°C. The turbidity of wells was observed and recorded.(bioMérieux, Inc.).

Extraction of DNA: Genomic DNA was extracted from the yeast with a rapid mini-preparation procedure [13]. To a 1.5-ml Eppendorf tube containing 500 µl of lysis buffer (400 mM Tris-HCl [pH 8.0], 60 mM EDTA [pH 8.0], 150 mM NaCl, 1% sodium dodecyl sulfate), a loopful of yeast colony was added aseptically by using a sterile loop, the tube was then left at room temperature for 10 min. After adding 150 µl of potassium acetate, pH 4.8 (5 M potassium acetate 60ml, glacial acetic acid 11.5ml, distilled water 28.5ml), the tube was vortexed briefly then centrifuged at >10 000 g for 1 min to remove the cellular debris and precipitated proteins. The supernatant was transferred to another 1.5-ml Eppendorf tube and centrifuged again as above. After transferring the supernatant to a new 1.5-ml Eppendorf tube, an equal volume of isopropyl alcohol was added. The tube was mixed briefly by inversion, centrifuged at >10, 000 g for 2 min and the supernatant was discarded. The resultant DNA pellet was washed in 300 µl ethanol 70% v/v. After centrifuging at 10 000 g for 1 min, the supernatant was discarded [14]. The DNA pellet was added to EZ-10 Spin Columns (Bio Basic Inc.)and centrifuged at >10, 000 g for 10 min. DNA was eluted in 50 µl of 1X TE buffer and stored at -20°C.

PCR Amplification: The PCR was used to amplify intergenic spacer regions (ITS) of gene encoding 5.8 S rDNA with primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC-3') (Willowfort.co.uk.). A reaction volume of 50 µl contained 25 µl master mix PCR [0.16 mM each deoxyribonucleoside triphosphate, 5 µl Taq DNA polymerase buffer, 2.5U Taq DNA-dependent DNA polymerase (intron master mix (i-TaqTM)), 0.2 µM each primer and 1µg genomic DNA. Reaction mixtures were subjected to initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 1 min, primer annealing at 50°C for 1 min and elongation for 2 min at 72°C. Final extension step was performed at 72°C for 10min. Negative control was carried outusing sterile deionized water instead of template DNA. 1.5% red safe stained agarose gel was prepared in 1X TBE buffer (Tris base/ boric acid/ EDTA). Agarose gel electrophoresis was run at 100 volt and the resulting bands were visualized by UV illumination. PCR products were stored at -20°C until used [15].

RFLP: In an Eppendorf, 8 µL from each PCR product were separately added to MspI and Hae III enzymes (New England Biolab NEB), 2 µL of 10X NEBuffer and 0.5 µL of the restriction enzyme (10 U/µL). The digested fragments were analyzed on 2.0% agarose gel [16].

RESULTS

Traditional Identification: 180 urine samples revealed 138 isolates suspected to be candida. 79 out of 133 appeared to be Candida albicans. 25 out of 133 isolates showed the characters of Candida glabrata, 14 isolates were Candida tropicalis, 7 isolates were Candida parapsilosis and 9 isolates were Candida Krusei.

Table 1: Results of traditional dentification

Species	Number of isolates	Percentage
Candida albicans	75	54.0
Candida glabrata	25	18.0
Candida tropicalis	18	13.0
Candida parapsilosis	4	3.0
Candida Krusei	16	12.0

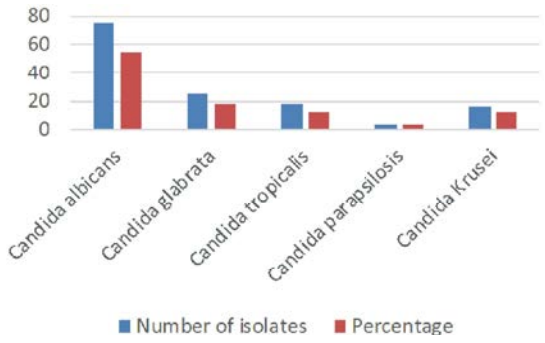


Chart 1: Results of traditional identification

Table 2: Results of molecular identification

Species	Number of isolates	Percentage
Candida albicans	82	59.0
Candida glabrata	26	19.0
Candida tropicalis	14	10.0
Candida parapsilosis	10	7.0
Candida Krusei	8	5.0

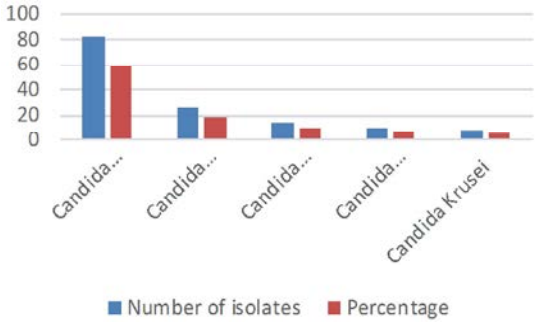


Chart 2: Molecular identification

Fig. 1: Candida albicans on SDA

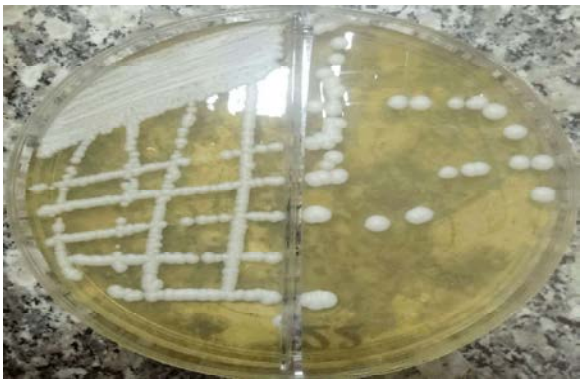


Fig. 2: Candida Glabrata and tropicales on SDA

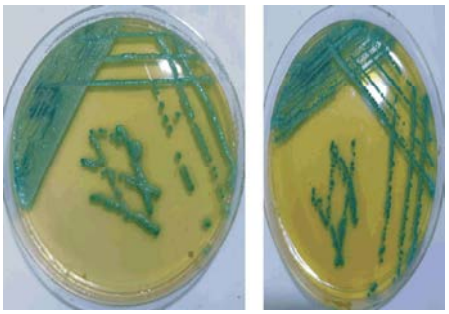


Fig. 3: Candida Albicans on CHROMagar Candida

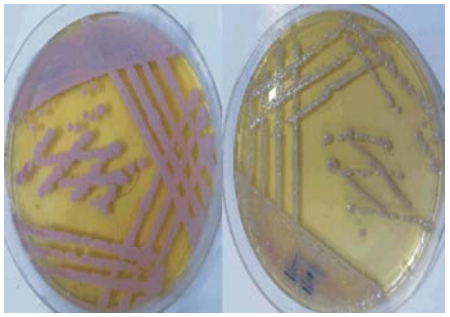


Fig. 4: Candida Krusei on CHROMagar Candida

Fig. 5: Candida Glabrata on CHROMagar Candida

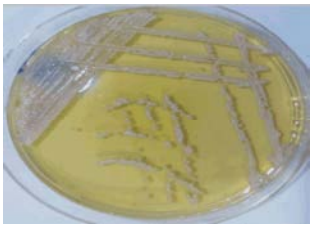
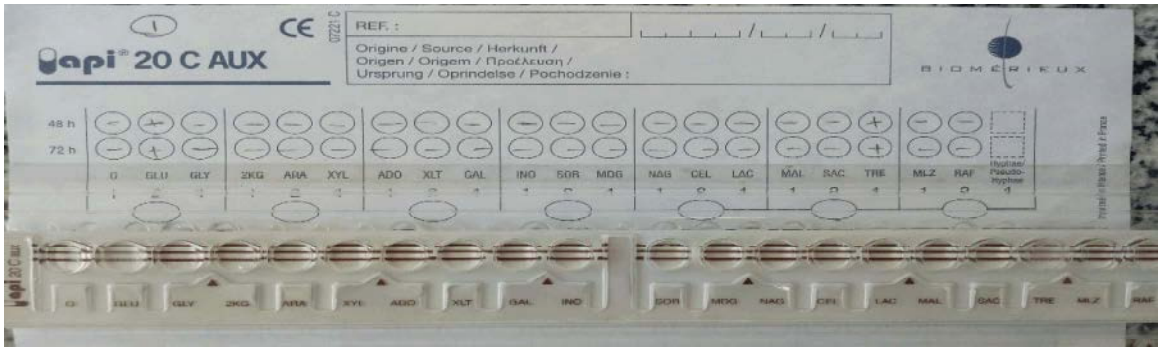
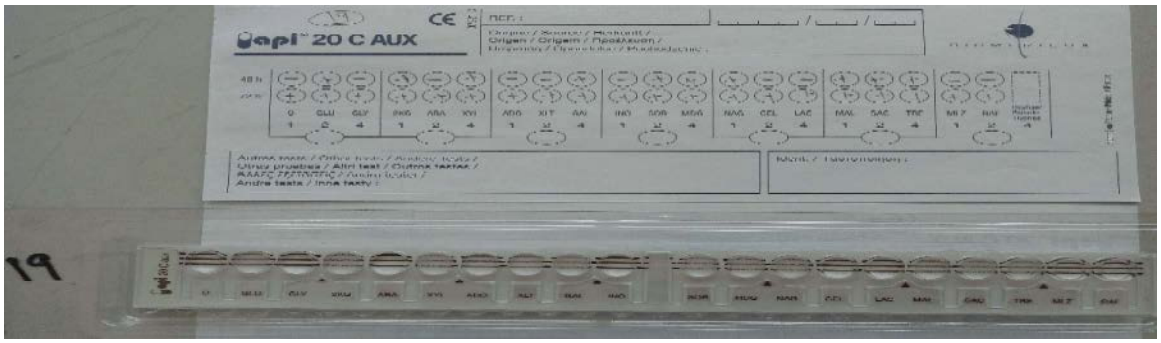


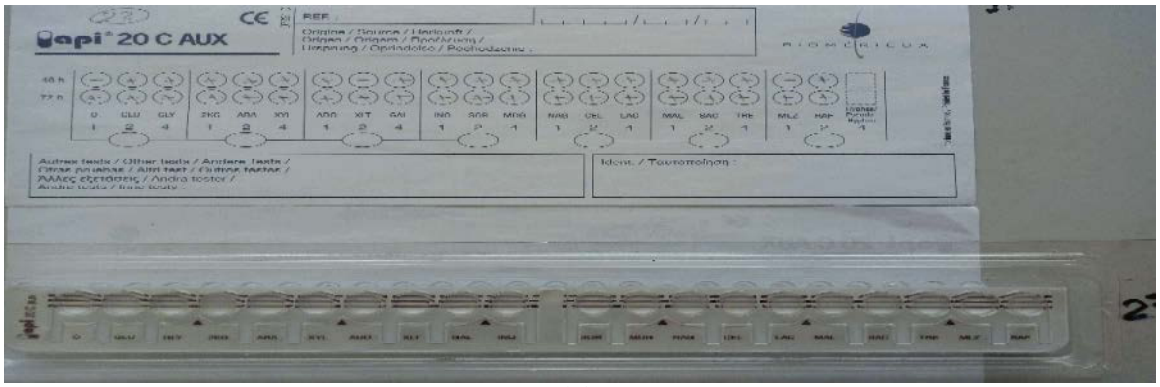
Fig. 6: Candida parapsilosis on CHROMagar Candida



(A)



(B)



(C)

Fig. 7: API 20 C: (A) Candida albicans by 96%, (B) Candida glabrata by 94%, (C) Cryptococcus by 73%

Fig. 8: Candida Albicans forming germ tube

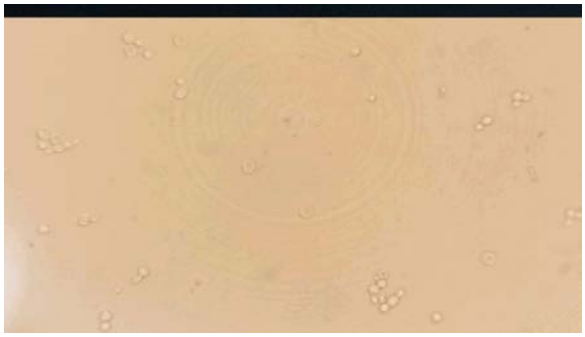


Fig. 9: Candida tropicalies negative for germ tube

Table 3: PCR products of (ITS1-ITS4)

Candida species	Size of (ITs1-ITs4)
C. Albicans	535
C. Glabrata	871
C. Tropicalies	524
C. Krusei	510
C. Guilliermondii	608
C. Parapsilosis	520

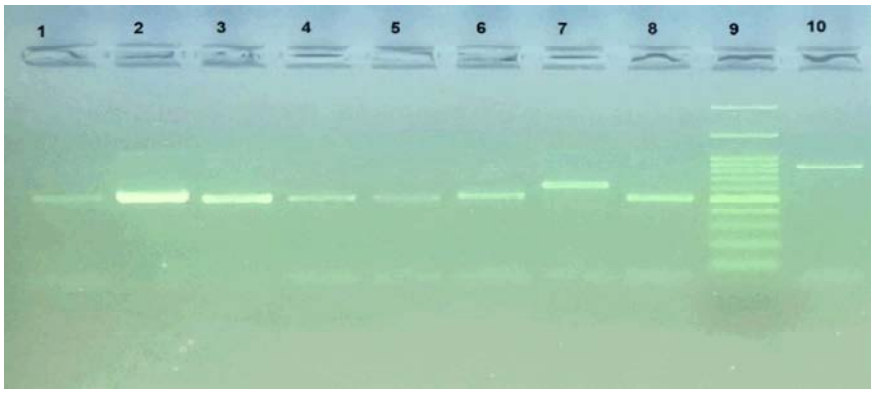


Fig. 10: PCR products from six *Candida species*. Lanes 1, 2, 3, 4, 5, 6, 8 arranged from 510-535 suspected to *C. albicans, C. tropicalis, C. krusi* and *C. parapsilosis*, Lanes 7 suspected to *Guilliermondii,* Lanes 9: DNA ladder 100 bp, Lanes 10 suspected to *C. glabrate*

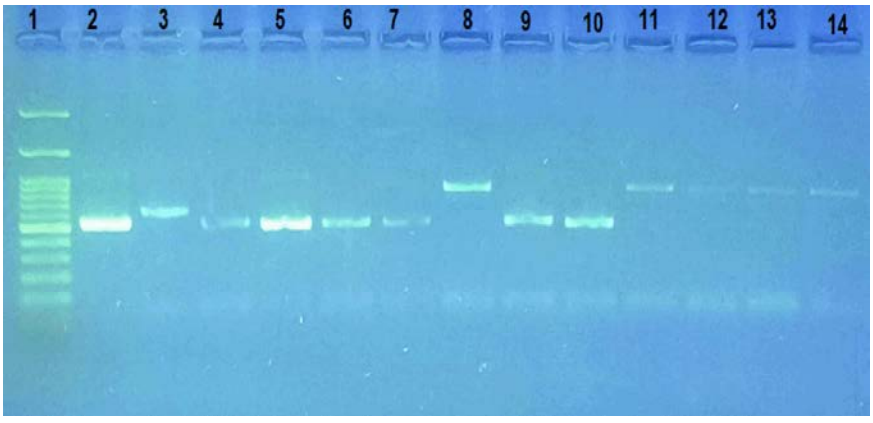


Fig. 11: PCR products from six *Candida species*. Lanes 1: DNA ladder 100 bp, Lanes 2, 4, 5, 6, 7, 9 and 10 arranged from 510-535 suspected to *C. albicans, tropicalies, C. krusi and C. parapsilosis*, Lanes 3 suspected to *C. guilliermondii,* Lanes 8, 11, 12, 13 and 14 suspected to *C. glabrate.*

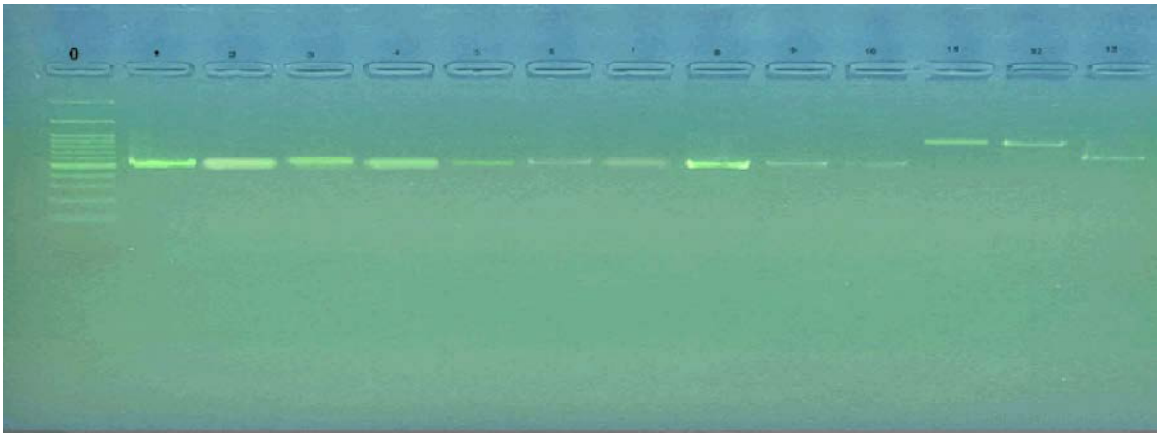


Fig. 12: PCR products from six *Candida species*. Lanes 0: DNA ladder 100 bp, Lanes 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 arranged from 510-535 suspected to *C. albicans*, *tropicalies*, *krusi* and *C. parapsilosis*, Lanes 13 suspected to *C. guilliermondii*, Lanes 11 and 12 suspected to *C. glabrate*

Table 4: Digestion with Msp I results

Species	Size by bp	No. of segmented
C. albicans	297, 238	Two band
C. glabrata	557-314	Two band
C. tropicalies	340-184	Two band
C. krusei	261, 249	Two band
C. guilliermondii	608	Un cut
C. parapsilosis	520	Un cut

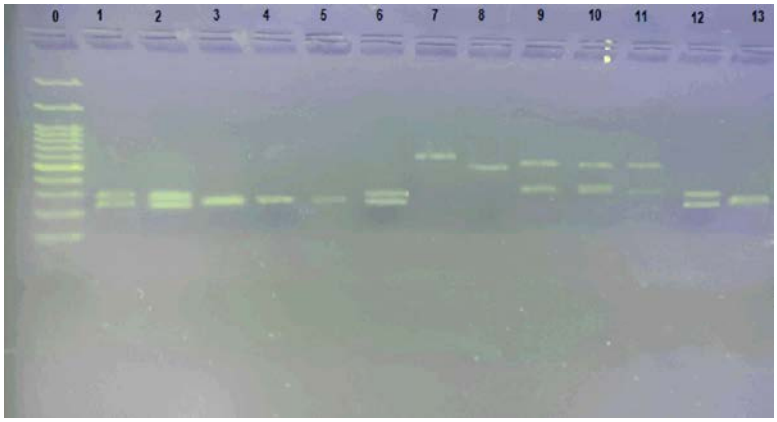


Fig. 14: Restriction digestion of PCR products of Candida strains with enzyme Msp I. Lanes 0: DNA ladder 100 bp, Lanes 1, 2: Candida albicans, Lanes 3, 4, 5and 12: Candida parapsilosis, Lanes 6 and 12: candida krusi, Lanes 7: candida guilliermondii, Lanes 8: Candida tropicalies and Lanes 9, 10 and 11: candida glabrata.

Table 5: Digestion with Hae III results

Species	Size by bp	No. of segmented
C. Albicans	430-90	Two band
C. Glabrata	650, 200	Two band
C. Tropicalies	524	Three band
C. Krusei	380, 90, 40	Three band
C. Guilliermondii	608	Un cut
C. Parapsilosis	390, 110, 40	Un cut

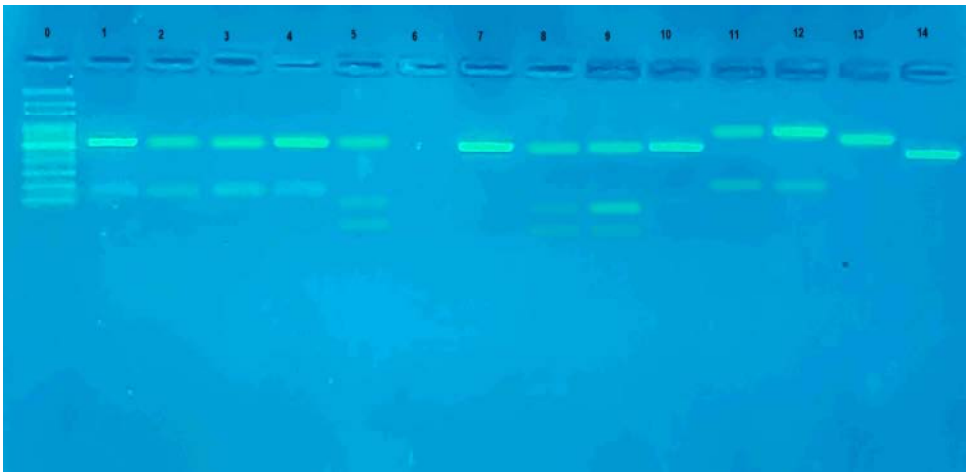


Fig. 15: Restriction digestion of PCR products of Candida strains with enzyme Hae III. Lanes 0: DNA ladder 100 bp, Lanes 1, 2, 3 and 4: Candida albicans, Lanes 5: Candida parapsilosis, Lanes 8 and 9: candida krusi, Lanes 10 and 14: candida tropicalis, Lanes 11 and 12: candida glabrata,

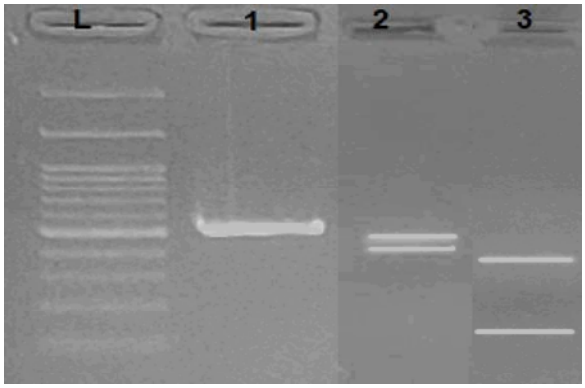


Fig. 16: Explains the rapid, accurate and reliable methods for identification of Candida albicans, Lane L: DNA ladder 100 bp, Lane 1: PCR products of amplification of 5.8s region of rDNA, Lane 2: restriction product of PCR product with Msp I, Lane 3: restriction product of PCR product with Hae III.

Molecular Identification: Upon carrying out molecular identification, 81 out of 133 isolates were identified as Candida albicans, with a percentage of 61.6. Candida glabrata was represented by 23 isolates (17.0%). 12 isolates exhibited the characters of Candida tropicalis with a percentage of 9.0, 10 isolates were identified as Candida parapsilosis (7.4) and 7 isolates Candida Krusei (5.0%).

DISCUSSION

Traditional identification including morphotyping and biotyping. Starts from macroscopically morphotyping using SDA and CHROMagar *Candida*. Colonies on SDA of different isolates appeared the same as creamy color of different species. CHROMagar can identify three species of *Candida* namely *Candida albicans*, *Candida tropicalis* and *Candida krusei*on the basis of colonial color andmorphology. The specificity and sensitivity of CHROMagar *Candida* for *C. albicans* are calculated as 99%, for *C. tropicalis* 98% and *C. krusei* 100% [17]. Our results of identification of the rest of Candida species using CHROMagar candida were consistent to those of Ilze Messeir *et al*. who stated that CHROMagar Candida is not beneficial for detection of other Candida species because of limited chromogenic substance to differentiate the rest of candida species [18]. Microscopic morphotyping using Gram staining for all *candida* species revealed Gram positive cocci with or without budding. Upon carrying out germ tube test, only *Candida albicans* gives positive results demonstrated microscopically as protruded tubes from the yeast cell. *However, Atta Yazdanpanah et al.* stated that *C. dubliniensis* and *C. africana produced germ* tube *as well* [19]. API 20C as a biotyping technique is a powerful biochemical assay for

Table 6: Traditional identification vis Molecular identification

	Traditional identification		Molecular identification	
Species	Number of isolates	Percentage	Number of isolates	Percentage
Candida albicans	75	54.0	82	59.0
Candida glabrata	25	18.0	26	19.0
Candida tropicalis	18	13.0	14	10.0
Candida parapsilosis	4	3.0	10	7.0
Candida Krusei	16	12.0	8	5.0

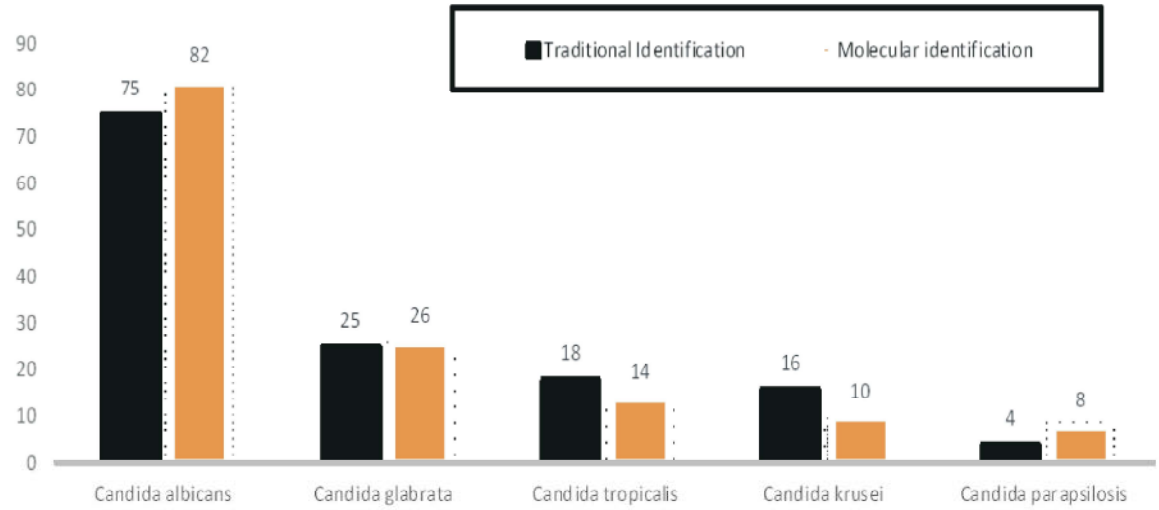


Chart 3: Comperhansive between traditional and molecular identification according to number of isoletes

discrimination among fungal isolates. Rama Ramani, *et al*. found that API 20C was very accurate for differentiation among Candida species with 93 % accuracy [20]. However, Maria Willemsen, *et al*. stated that the accuracy of API 20°C when using for the differentiation among Candida species was 60.1% and 69.4% after 24 and 48 hours incubation respectively [21]. In this study API 20°C was not reliable for the identification of *Candida* species.

Molecular identification of fungal isolates to the species level attracts many scientists because of its reliability, accuracy and rapidity. Highly conserved regions such as 5.8 S rDNA are commonly used for molecular identification among microbial species. ITS1-ITS4 region is used as a discriminatory clue for identification of candida species [22]. PCR products of ITS1- ITS4 were digested individually with two different restriction enzymes MspI and Hae III. The restriction patterns obtained for five strains were identical with the restriction pattern of *C. albicans* and one was identical with the restriction pattern of *C. krusei* [23]. The above mention results showed that molecular identification of candida species can lead to accurate, rapid and reliable identification and discrimination of candida isolates to the species level.

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