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Multiplex PCR identification of eight clinically relevant Candida species isolated from groin Fungal Infections among Egyptian patients

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Abstract: *The prevalence of fungal infections has raised in recent years due to an increasing population of immunosuppressed patients, intensive immunosuppressive chemotherapy, increasing awareness of fungal infections, and the widespread use of broad-spectrum antibiotics .Therefore, in the last few years, the development of novel diagnostic methods has been regarded a critical issue. A multiplex PCR strategy allowed the identification of 8 clinically relevant yeasts of the Candida genus, namely C. albicans, C. glabrata, C. parapsilosis, C. tropicalis, C. krusei, C. guilliermondii, C. lusitaniae and C. dubliniensis. This method is relied on the amplification of two fragments from the ITS1 and ITS2 regions by the combination of 2 yeast-specific and 8 species specific primers in a single PCR reaction. This novel method provides a clinical diagnostic procedure with direct applicability.In addition to, it allows specific differentiation of individual Candida species within polyfungal samples.*

Keywords:*Candida, candidemia, diagnostic, identification, PCR, multiplex.*

I. Introduction

Within the last two decades, the opportunistic fungus *Candida albicans* was the pith of cogent medical interest since it still engenders substantial morbidity with significant mortality rates especially in immunosuppressed patients as HIV positive persons, cancer patients and transplant recipients among others [1,2].Candidiasis infection vary from superficial mucosal local lesions to a disseminative disease[3].The polymorph *C.albicans* fungus grows either in a yeast phase or as filamentous phase (hyphae). These two types of morphology could be present in infected tissues, thus it's possibly overt that both types contribute to the pathogenesis of the microorganism[4,5].*Candida*spp are the most common causative agent in fungal contagions[6],representing a range of invasive life- threatening to non-life-threatening mucocutaneous diseases. *Candida albicans* among *Candida*spp is the most frequently common pathogenic infectious agent. It is a commensal dimorphic yeast that colonizes the skin, reproductive and gastrointestinal tract.Non *C.albicans* species are emergent fungal pathogens that as well colonizing human mucocutaneous surfaces[7].Mucocutaneous candidiasis is categorized into non genital disease and genitourinary disease, oropharyngeal manifestation usually common in non-genitourinary, frequently diagnosed in HIV patients[8].Whereas, genitourinary candidiasis include vulvovaginal candidiasis in females, balanitis and balanoposthitis in males and candiduria in both sexes[9].Although *C.albicans* is the most frequent and clinically related genus of the species, however there has been a substantial tendency towards the egression of species other than C.albicans with a particular step up in *C.glabrata* frequency[10].But a lesser tendency of *C.parasilosis* and *C.tropicalis*[11].The previously mentioned non *C.albicans* species are resistant to common therapeutic antifungal chemicals, where exact identification tests are crucial for formation and establishment of consequential antifungal therapy[11].The standard normal criterional tests for candidiasis diagnosis include culture and histopathology techniques that both lack accuracy of detection . Culture identification is relied on the visual changes that may occur in either microscopic, macroscopic morphology or pigmentation. Atypical growth may frequently take place causing confusion during the identification process, also false negative culture is possible to be noticed[11]. These methods have circumscribed specificity and low sensitivity[12].As in case of blood culture that is positive for fewer than 50% of patients with hepatosplenic candidiasis[13],are seldomly positive in patients suffering invasive aspergillosis[14]. Moreover, these procedures for identification purpose are time consuming and may cause inconclusive results, as VITEK and ID32C systems that necessitate many days before the biochemical reactions can by analyzed or interpreted[19].Therefore an alternative rapid and highly specific diagnostic procedure has been a tempting inspiration [17].Recent several molecular and immunological attempts for highly specific fungal pathogen identification include sensing and detecting the antigen, antibody or DNA.Antibody detection in bone marrow transplant(BMT) patients still remains of limited clinical diagnostic value due to the uncertain and unpredictable humoral responses[15].However molecular identification approach especially multiplex PCR and realtime PCR assay for fungal nucleic acids detection

showed accurate and specific fungal identification plus many laboratory advantages[18].It can be applied to a variety of specimens, embracing and encompassing multiple fungal genera in one reaction showing higher specificity than common culture based methods[18].However, in spite of its high sensitivity, it demands specific and expensive equipments This molecular reaction can detect candidemia with elevated sensitivity based on fungal ribosomal genes as DNA targets in PCR reactions[20].Buchman et al were the first to certify and demonstrate the specific *Candida albicans* detection possibility by PCR amplification of the lanosterolalpha de-methylase (L1A1)gene[20].At the molecular identification species level for medically important yeasts identification, the highly variable sequences of internal transcribed spacer regions (ITS1) and (ITS2) that both are flanked by the relatively conserved coding regions of 28S, 5.8S and 18S nuclear ribosomal (rRNA)genes were utilized in different PCR formatting [21].For ITS1 and ITS2 in vitro amplification by m-PCR technique Integrating 8 Candida species specific primers and two yeast specific primers in one single PCR reaction is performed[21].

This study represents a comparison of identification between common phenotypic culture methodsandsimple rapid multiplex PCR reaction foreight clinically relevant yeasts of the *Candida* genus namely *C. albicans, C. glabrata, C. parapsilosis, C. tropicalis, C. krusei, C. guilliermondii, C. lusitaniae and C. dubliniensis.*Giving rise to amplicons with different sizes with a detection limit 2.15±.25 cells/ml with advantage of incorporating whole yeast cells in one single reaction.

Aim of the work:

To determine the range of candida species detected by PCR technique applied on patient specimens and to compare this technique with conventional culturetechnique which detects the phenotypic criteria of candida on culture media.

II. Materials &Methods

Twenty patients with intertrigo were randomly selected from the out-patient clinic of Dermatology, Andrology and Venereology Department in Ain Shams University Hospital. An informed consent to participate was obtained from all patients. For all patients, the following was done: 1) A detailed history including age, sex, underlying disease and prior antifungal treatment. 2) Thorough general and local clinical examinations were done to all cases to search for any associated conditions .3) Swab collection. 4) Staining with Gram stain and for direct film examination. 5) Inoculation in Sabouraud's dextrose agar (SDA) and Brilliance candida agar (chromogenic media) for 48-72h at room temperature .6) Identification of isolates: the yeast like colonies were identified by classical methods, while differential medium chromogenic media was used to identify candida species by colony morphology and pigmentation according to the by chromogenic identification methods [22].
6) Strains were isolated and stored in glycerol for PCR identification.

Primer design

Two Yeast specific universal primers UNI1(5`-GTCAAAC TTGGTCATTTA-3`) and UNI2 (5`-TTCTTTTCCTC CGCTTATTGA-3`) were incorporated to in vitro amplify the internal transcribed spacer regions 1 (ITS1) and 2 (ITS2), integrating the 5.8S rRNA of the most common yeast pathogens[23]. In addition, the species-specific primers Calb, Cgla, Ckru, Cpar, Ctro, Clus, Cgui and Cdubwere designed based on the sequence data for the ITS1 and ITS2 regions of the reference strains and of all clinical isolates from the Candida genus available in the EMBL/GenBank databases. The sequences were compared using the DNAMAN for Windows software (Lynnon Corporation, Quebec, Canada) in order to design primers to specifically amplify DNA from (*C. albicans, C. glabrata, C. krusei, C. parapsilosis, C. tropicalis, C. lusitaniae, C. guilliermondii and C. dubliniensis)* respectively (Table 1).

DNA Isolation

For DNA extraction, cells were grown overnight in YEPD medium at 26°C on a mechanical aerated shaker (150 rpm) [24].Centrifugation of cells for harvesting at 3,000 for 5 min[24]then the pellet suspended in 200 ml of lysis buffer (2% Triton X-100, 1% SDS, 100 mMNaCl, 10 mMTris-HCl, and 1 mM EDTA, pH 8.0). Disruption of cells by 200 ml of 0.5 mm diameter glass beads and 200 ml of chloroform/phenol (1:1) were added and the tubes were shaken for three 60-second intervals with periods of cooling on ice[24]. Cell remains were eliminated centrifugation at 3,000 g for 5 minutes,the supernatant was collected and 1 ml of cold isopropanol was added prior to mixing by inversion. Then Centrifugation of tubes at 3,000 g for 3 minutes where the sediment was suspended in 400 ml of TE buffer (100 mMTris-HCl, 1 mM EDTA, pH 8.0)[25]. A 5-min treatment with RNase A (1 mg/ml) at 37°C was then carried out before adding 10 ml of 3M sodium acetate. For further DNA precipitation,1ml of isopropanol was added, then mixing by inversion for centrifugation. Finally, the sediment was air-dried and the DNA suspended in 50 ml of ultrafiltered water. DNA content and

purity were determined by spectral photometry at 260 and 280 nm and diluted to a 100 ng/ml final concentration[25].

Table 1 Universal and species-specific primers used in Candida species amplification and size of fragments visualized under agarose gel electrophoresis

Species	Primer name	Sequence (5'-3')	Amplicon size (bp)*
Clinically relevant yeasts	UNI1 UNI2	GTCAAACTTGGTCATTTA TTCTTTTCCTCCGCTTATTG	Trost et al., 2004 [22]
C. albicans	Calb	AGCTGCCGCCAGAGGTCTAA	583/446
C. glabrata	Cgla	TTGTCTGAGCTCGGAGAGAG	929/839
C. krusei	Ckru	CTGGCCGAGCGAACTAGACT	590/169
C. tropicalis	Ctro	GATTTGCTTAATTGCCCCAC	583/507
C. parapsilosis	Cpar	GTCAACCGATTATTTAATAG	570/370
C. guilliermondii	Cgui	TTGGCCTAGAGATAGGTTGG	668/512
C. lusitaniae	Clus	TTCGGAGCAACGCCTAACCG	433/329
C. dubliniensis	Cdub	CTCAAACCCCTAGGGTTTGG	591/217

In QIAamp mini spin columns with 2 ml collection tubes, centrifugation at 5,000 g for 1 min then washing twice with 500 ml of AW buffer. The columns were then washed twice with 50 mM EDTA and twice with AW buffer in order to chelate PCR inhibitory factors. DNA was eluted with 100 ml of previously heated AL buffer and kept at -20°C until PCR. ATL lysis buffer, AL buffer, AW buffer, proteinase K and the spin columns were purchased from Qiagen, Hilden, Germany.

PCR Amplification

Multiplex PCR amplification reaction was carried out in a 20 ml volume consisting of 0.8_PCR buffer [160 mM (NH4)2SO4, 670 mMTris-HCl (pH 8.8)], 3.5 mM MgCl2, dNTP mixture (200 mM each), primer mixture (UNI1 and UNI2, 0.55 mM each; Cgui, 0.05 mM; Calb and Ckru, 0.15 mM each; Cgla, Ctro and Clus, 0.2 mM each; Cpar, 0.3 mM; Cdub, 0.4 mM), 1 U TaqDNApolymerase and 50 ng genomic DNA template, with the remaining volume consisting of sterilized water[21]. The DNA template amout used quantified by spectrophotometry[20]. For colony-PCR, part of a single colonywas suspended directly in the PCR mixture with a sterile toothpick. PCR was routinely performed in a BiometraTpersonal (WhatmanBiometra, Goettingen, Germany) thermal cycler under the following cycling conditions: 40 cycles of 15 s at 94°C, 30 s at 55°C, and 45 s at 65°C, after a 10-min initial period of DNA denaturation and enzyme activation at 94°C[21]. Additionally, multiplex PCR reactions were performed in four different thermal cyclers: iCycler and MyCycler (Bio- Rad, CA, USA), Primus 96 (MWG Biotech AG, Ebersberg, Germany) and GeneAmp PCR System9600 (Perkin-Elmer, MA, USA) in order to confirm PCR reaction reproducibility. Negative control reactions were carried out simultaneously with each test run by substituting the template DNA with sterilized water inthe PCR mixture[27]. 10 ml aliquots of each amplification product were separated by electrophoresis in a 2% agarose gel. Ethidium bromide staining (0.5 mg/ml) allowed the visualization of DNA fragments witha digital imaging system (Alpha Innotech Corporation, CA, USA) and species identification was possible by comparison with a 100-bp DNA ladder (Fermentas International Inc., Ontario, Canada).

Statistical analysis: statistical presentation and analysis of the present study was conducted using the mean standard error, unpaired bivarientt.test and chi-square using SPSS program version:22.0.0.0.

III. Results

A total number of 20 patients were enrolled in thisstudy.The patients were 12 male (60) and 8 females (40). With mean age 31 range (0.65-39).

Results of culture on chromogenic media showed 11 positive samples for candida growth on chromogenic media, 2 samples were (*c.alb+c.kreusi*) (10%) indicated by green and pink -brown color change on chromogenic media respectively(figures 2,5), 1 (*c.alb +c. parapsilosis*) (5%) indicated by green and brown color change respectively(figures2,6) , 1 (*c.alb + c.tropicalis*) (5%) indicated by green and dark blue color change(figure3) , 1 (*c.alb + c.glabrata*) (5%) indicated by green and yellow(figure3), 2 (c.kreusi) (10%)(figure 5), 1 (*c.parapsilosis*) (5%)(figure 6), 1*(c.tropicalis*) (5%)(figure 4) and 2 (*c.glabrata*) (10%)(figure 3).The rest of samples (9) were negative(45%)as they showed no color change on chromogenic media .

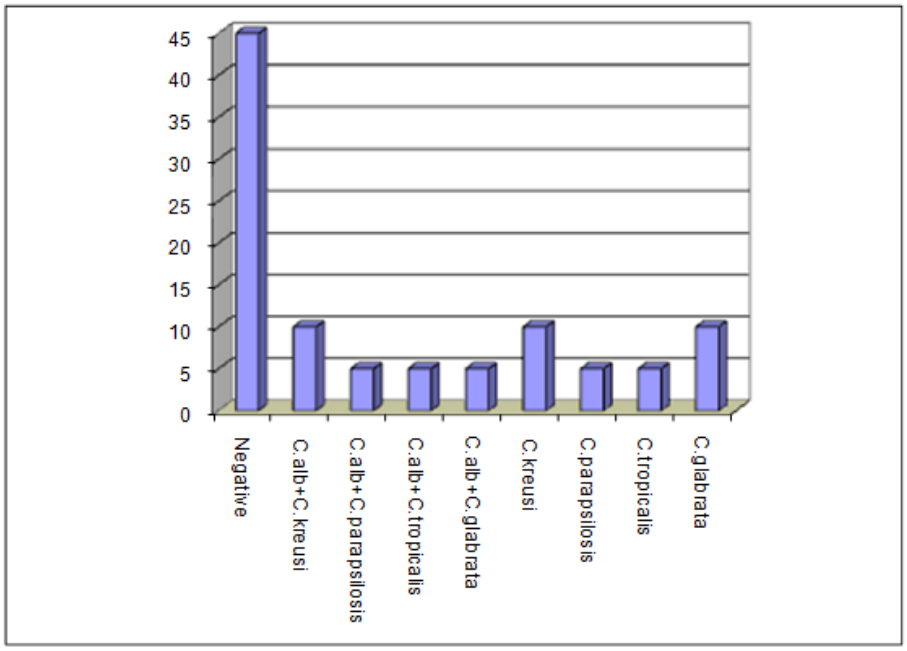

Fig. (1): Results of culture on chromogenic media.

Fig. (2): Growth of Candida Albicans (green) on brilliance agar

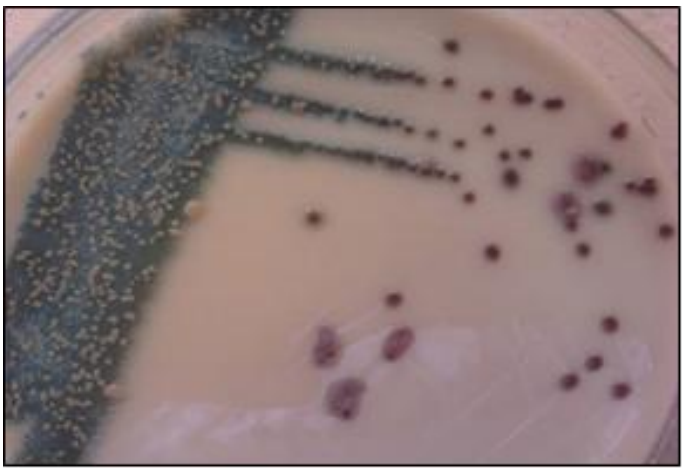

Fig. (3): Growth of Candida Tropicalis(dark blue) with glabrata (light brown) and Albicans (green) on brilliance agar.

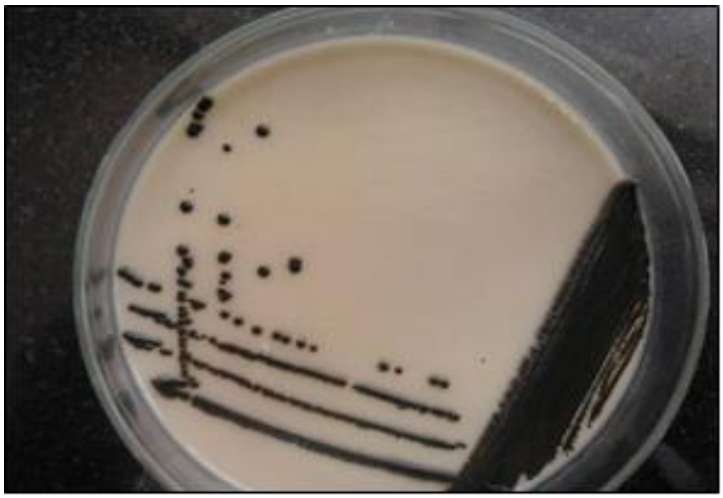

Fig. (4): Growth of Candida Tropicalis(dark blue) on brilliance agar

Fig. (5): Growth of Candida Krusei (pink)on brilliance agar.

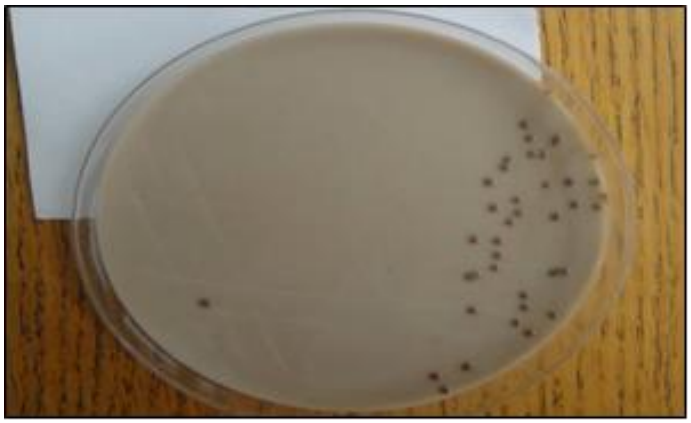

Fig. (6): Growth of Candida Parapsilosis(brown) on brilliance agar.

Results of PCR with incidence of each candida were *candida albicans* 7(38.9%), *candida kreusi* 4(22.2%), *candida parapsilosis* 2(11.1%), *candida tropicalis* 1(5.6%) and *candid glabrata* 4(22.2%)

Table(2): Results of PCRwithincidenceof each*candida* species

ResultsonPCR	**Frequency**	**Percent**
C.albican	7	38.9
C.kreusi	4	22.2
C.parapsilosis	2	11.1
C.tropicalis	1	5.6
C.glabrata	4	22.2
Total	18	100.0

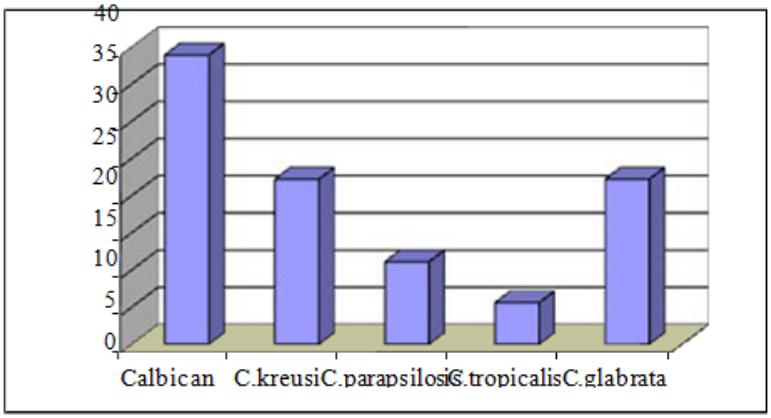

Fig.(7): Results of PCR withincidenceof eachcandidaspecies

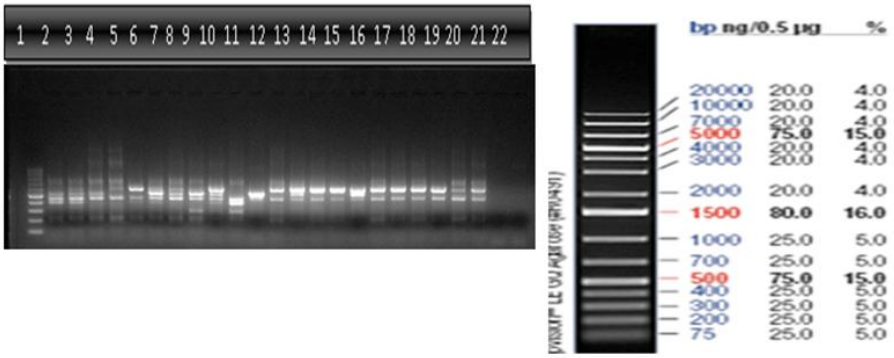

Fig. (8): Gel electrophoresis showing PCR products fragment size measured against GeneRuler™ 100 bp Plus DNA Ladder. According to fragment size *Candida spp*. Were identified as follows: Lane 1: *C. lusitanae*, Lane 2,3,4: *C. glabrata*, Lane 5-9, 11,12: *C.guillermondi*, Lane 10: *C.krusei*, Lane: 13-20:*C.albicans*, Lane 21: *C.tropicalis*, Lane 22: *C.parapsilosis*

Table(3): Comparison betweentheresultsobtainedby chromogenicmediaandPCRasregardidentificationof *candida* species

	ResultsbyChromogenic agar		**ResultsbyPCR**		**Chi-squaretest**	
	No.	**%**	**No.**	**%**	**X^2**	**P-value**
Calbican	5	31.25%	7	38.89%	0.694	0.952
C.kreusi	4	25.00%	4	22.22%		
C.parapsilosis	2	12.50%	2	11.11%		
C.tropicalis	2	12.50%	4	5.56%		
C.glabrata	3	18.75%	18	22.22%		
Total	16	100.0		100.0		

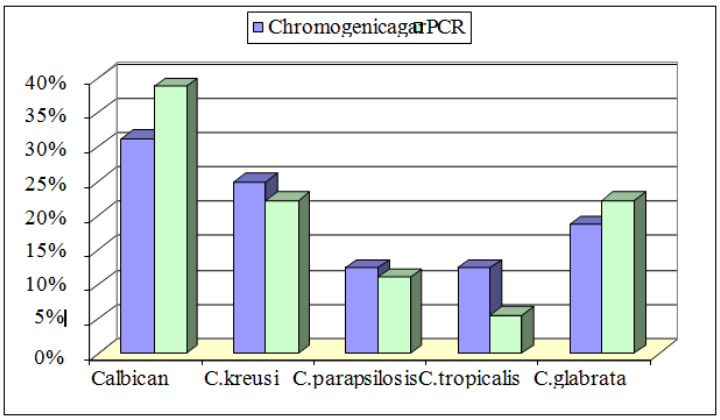

Fig.(9): Comparisonbetweentheresultsobtainedby chromogenic mediaandPCR as regardidentificationof candidaspecies.

No significant statistical difference was found between the results of chromogenic and PCR as regard identification of candida species (P>0.05) and showed good correspondence with chromogenic media (concordance value 79.1%). Both have nearby ability to discriminate *Candida* species

Table(4): ConcordanceratebetweenPCRandchromomeric agar

	ResultsbyPCR		**ResultsbyChromogenic agar**	
	No.	**%**	**No.**	**%**
Calbican	7	38.89%	5	31.25%
C.kreusi	4	22.22%	4	25.00%
C.parapsilosis	2	11.11%	2	12.50%
C.tropicalis	1	5.56%	2	12.50%
C.glabrata	4	22.22%	3	18.75%
Concordancevalue	79.1%			
Discrepancy value	20.9%			

Table(5): Sensitivityandspecificityforchromogenicagarin comparisonwithPCRresults

		C.albican	***C.Kreusi***	***Parapsilosis***	***C.tropicalis***	***C.glabrata***
Chromogenicagar	Sensitivity	71.4	100.0	100.0	100.0	75.0
M-PCR	Specificity	100.0	100.0	100.0	94.4	100.0

IV. Discussion

The cornerstone of this study is a comparison between standard phenotypic methodology and multiplex PCR assay for specific identification and sensitive detection of eight relevant *Candida* species namely *C. albicans, C. glabrata, C. parapsilosis, C. tropicalis, C. krusei, C. guilliermondii, C. lusitaniae and C. dubliniensis*in polyfungal samples for accurate clinical diagnostic and therapeuticpurposes. Phenotypicculture techniques for morphological identification relied on the visual alterations of the micro and macroscopic features of the polymorph fungus as well as pigmentation.Multiplex PCR method based on in vitro amplification of the fungal ribosomal genes for molecular identification purpose.The second approach is carried out in a single multiplex PCR reaction coexisting 8 clinically relevant Candida species isolated from 20 swab samples randomly collected from out patient clinic of dermatology and venereology Department, in Ain Shams University Hospitals. The patients were not receiving any topical or systemic treatment for their conditions, and the present work was approved by ethical committee. They all were subjected to history taking, clinical examination, specimen collection (lesional swab), gram staining and culture for all collected samples on sabouraud dextrose agar and selective chromogenic media (brilliance candida agar). Then all the positive culture media on chromogenic media was subjected to PCR reactions.The patients were 12 male (60) and 8 females (40) With mean age 31 range (0.65-39).

The suggested m-PCR reaction based on the combined usage of universal primers UNI1 and UNI2 and 8 Candida species-specific primers to in vitro amplify the internal transcribed space regions ITS1 and ITS2 (tab.1), including the 5.8S rRNA region[21].Taking advantage of the high copy number of r-RNA genes, different lengths of ITS regions and the high variability of these regions among *Candida* species.Phenotypic Results obtained from differential chromogenic media after 28 days showed characteristic pigmentations(fig.2,6).On the other handm-PCR products obtained and identified within as little as 5 hours(tab.2) , reveled the characteristic band pattern(fig.8),discriminating the different species present in polyfungal samples(fig.7).Comparison between both results as regard identification of *Candida* species(fig.9),showed no significant statistical differences where (P>0.05) (tab.3).The concordance rate between PCR and chromogenic agar was (79.1%),however many discrepancies was also recorded in such comparison(20.9%) (tab.4).Also sensitivity and specificity from chromogenic agar in comparison with PCR products showed nearby ability to discriminate *Candida* species(Tab.5).

In this study 20 swab samples yielded poor *candida* species outcome, especially *C.Tropicalis* that was one sample identified by m-PCR and two identified by chromogenic agar representing (5.56% ,12.50 %) respectively(fig.1)&(tab.2). So for further investigations, it's highly recommended to use cultural isolates rather than swab samples for increased fungal specimens for better confirmation and deep understanding. Although both techniques showed nearby discrimination of *Candida* species,however m-PCR reaction is highly specific and moresensitive, but it still an expensive equipment not readily accessible to diagnostic laboratories in contrast to common culture methods. M-PCR molecular technique has plenty of laboratory advantages rather than phenotypic methodology as we noticed. It's time saving, few hours for the results to come up, in contrast to standard morphological, cultural and biochemical features that takes 28 days. Highly specific with sensitive detection limit 2.15±.25 cells/ml advantaging the occurrence possibility offalse negative culture results. Moreover,wholeminor yeast cells could be directly incorporated in the reaction, easily implemented, cost effective since no need for either the restriction enzymes or DNA probes[23]. In addition, it can be appliedto a

variety of specimen types testing several clinical samples for rapid identification and consequently provides accurate therapeutic clue.

Conclusion: Both PCR and chromogenic agar media showed good correspondence in Candida species identification (concordance value 79.1%). Also multiplex PCR was rapid and effective in the identification of Candida species allowing the detection of more than one species at the same time.

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