

Evaluation of Rapid Molecular Identification of Clinically Important *Candida Spp* Isolated From Immuno-Compromised Patients Using RF-PCR

Ayman A. Allam and Ihab M. Salem

Microbiology and Immunology and Internal Medicine Departments, Faculty of medicine, Zagazig University. egyayman66@yahoo.com

Abstract: *Candida species* have become an important cause of nosocomial infection especially in immunocompromised patients. Current phenotypic identification methods are time consuming so molecular methods were used for rapid identification of *candida spp*. The aim of this study was to evaluate restriction fragment polymerase chain reaction as a rapid and accurate method for identification of *Candida spp* isolated from immunocompromised patients. 170 different specimens collected from 120 immuno-compromised patients were subjected to standard fungal methods to isolate *Candida spp*. All candida isolates were subjected to API AUX to confirm phenotypic identification. DNA was extracted from all candida isolates and subjected to amplification using the ITS1 and ITS4 primer pairs. All amplicons were subjected to digestion directly and individually by the restriction enzyme *MspI*. The restriction products were checked using agarose gel electrophoresis. 52 (30.6%) out of 170 specimen were positive for *Candida spp*. The highest percentage of *Candida spp* were isolated from oral swabs (60%), followed by peritoneal dialysate (34%), urine (24%), sputum (23%) and the lowest percentage was from pus (10%). by the use of API 20 C AUX, the highest percentage of *Candida spp* isolated was *C albicans* {23 isolates (44.2%)}, followed by *C tropicalis* {13(25%)}, then *C glabrata* {6 (11.5%)}. One (1.9%) of each of *C krusei*, *C stellatoidea*, and *C kefyr* was isolated. *C albicans* was the most frequently *Candida spp* isolated from every specimen type. The intergenic spacer region was successfully amplified from all *Candida* isolates tested giving amplification product 510-871 bp. In all *Candida* isolates, Identification by RF PCR shows 86.5% agreement with API. Identification by RF PCR shows 100 % agreement with API identification in case of *C tropicalis*, *C glabrata*, *C krusei* and *C guilliermondi*. In case of *C albicans*, 96% (22 out of 23) agreement of RF PCR is shown in comparison to API. RF-PCR fails to identify isolates of *C kefyr*, *C lusitanae* and *C parapslosis* to species level. This study concluded that *Candida albicans* still the most important *Candida spp* affecting immunocompromised patients and Non *candida albicans* spp are emerging important pathogens. It also concludes that PCR RFLP using the restriction enzyme *MpsI* is a good rapid identification method that identifies the most important *Candida spp* isolated from immunocompromised patients and recommends further studies to develop new methods using different restriction enzymes to increase the range of identified *candida spp*.

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1. Introduction

Candida species have become an important cause of nosocomial infection⁽¹⁾. The incidence of disseminated candidiasis has increased in recent years because of a rise in the number of immuno-suppressed and postoperative patients⁽²⁾.

When immune system is weak (e.g., in immunocompromised patients as the result of cancer chemotherapy or HIV infection) or when the competing flora are eliminated (after antibiotic treatment), *C.albicans* colonizes and invades host tissues⁽³⁾

Candida albicans remains the most common cause of candidiasis, but other species are not uncommon⁽⁴⁾. Thus, early and accurate diagnosis of an invasive fungal infection is critical for timely and appropriate treatment⁽⁵⁾, perhaps improving chance

of survival especially in immunocompromized patients. Although current phenotypic identification methods as biochemical analysis, germ-tube examination, chlamydospore examination on rice infusion tween 80 agar and evolution of colonial morphology on chromogenic agar represent conventional and reliable approaches, they are time consuming and require 48 to 72 hrs or longer to give definitive identifications. So many molecular techniques as nested PCR, multiplex PCR, Taq-man PCR, light-Cycler PCR and fluorescent PCR are developed targeting to detect *Candida species* in a short period of time, with a high sensitivity and specificity⁽⁶⁻⁸⁾.

The purpose of this study was to evaluate restriction fragment polymerase chain reaction as a rapid and accurate method for identification of

Candida spp isolated from immunocompromised patients.

2. Material and Methods:

This case control study was conducted in Microbiology and Immunology and Internal Medicine Departments of Zagazig University Hospitals during the period from June 2010 to may 2011.

One hundred and twenty (120) immunocompromised patients were enrolled in this study. 30 patients with blood malignancy (leukemia and lymphoma) under treatment, 30 with solid malignancy under chemotherapy and radiotherapy, 30 with renal failure and 30 patients under chronic corticosteroid therapy.

Microbiological samples including 40 oral swabs, 10 pus swabs, 35 peritoneal dialysate, 25 midstream urine, and 30 sputum samples collected and cultured according to standard methods on Sabouraud's dextrose agar (SDA) with chloramphenicol and chlorhexamide (Oxoid) then incubated in the incubator at 37°C for 48 hours. 40 blood samples (5-10 ml) were aseptically withdrawn and inoculated in ready made blood culture bottles, incubated at 37°C for 5 days with daily subculture on SDA⁽⁹⁾. Any growth was subjected to gram stain, and any suggestive *Candida* isolate was subjected to germ tube testing and identification using API 20 AUX (Bionereux, Paris, France). Three colonies with characteristics of *Candida spp* were inoculated individually in yeast peptone dextrose (YPD, Sigma) broth and incubated at 30°C for 48 hrs. After this period, each culture was individually stored at -20°C with 20% glycerol. Only one sample of each isolate was used for all characterizations.

DNA extraction:

For DNA extraction, yeasts were grown on Sabouraud dextrose agar plates (Difco) at 37 °C for 24-48 hrs. A single colony was cultured overnight on YPD broth (1% yeast extract, 2% peptone, 2% dextrose) at 37 °C. DNA was extracted using the DNeasy Isolation Kit (Qiagen, USA). An A260/A280 ratio of 1.8-2.1 was considered acceptable.

PCR Conditions:

Primers: PCR amplification of ITS1-5.8S-ITS2 rDNA regions was achieved using the ITS1 (forward, 5'-TCC GTA GGT GAA CCT GCG G-3' and ITS4 (reverse, 5'-TCC TCC GCT TAT TGA TAT GC-3') primer pairs (Fermentans, Germany) which were described previously^(10,11).

Amplification conditions: PCR amplification was performed in a final volume of 50 µl. Each reaction consists of 2 µl templates DNA, 0.5

µl of each primer at 25 µM, 1.25 µl of dNTP at 5 mM, 0.5U Taq DNA polymerase (Roche Diagnostics, Germany) and 5 µl 10× PCR buffer. Amplifications were carried out in a thermal cycler (Perkin-Elmer cetus type 480). The amplification parameters consisted of 35 cycles of denaturation at 94°C for 1 min, primer annealing at 56°C for 1 min, extension at 72°C for 1 min. In the first cycle, the denaturation step was 94°C for 5 min and in the final cycle the final extension step was 72°C for 7 min.

Expected products of amplification are 510-871 bp (*C.albicans* 535 bp, *C glabrata* 871 bp, *C tropicalis* 524 bp, *C krusei* 510 bp, *C guilliermondii* 608 bp, *C parapsosis* 520 bp)^(10,12).

Restriction enzyme analysis:

A volume of 21.5 µL of PCR products were digested directly and individually by 10U (1µL) of the restriction enzyme *MspI* and 2.5µL related buffer (total reaction volume of 25µL) by 90 min incubation at 37 °C. Restriction fragments expected are *C.albicans* 297 and 238 bp; *C glabrata* 557&314 bp; *C tropicalis* 340&184 bp; *C krusei* 261&249 bp; *C guilliermondii* 371,155&82 bp⁽¹⁰⁾.

Agarose gel electrophoresis:

The resulting amplified products and restriction fragments were analyzed by 2.5% agarose gel. Electrophoresis gel was conducted in TBE buffer (0.1 M Tris. 0.09 M boric acid, 20 mM EDTA, pH = 8), at 100 V for 45 min. The gel was stained with 0.5 µg mL of ethidium bromide and examined by ultraviolet trans-illuminator.

3. Results:

As shown in table (1), 52 (30.6%) out of 170 specimen were positive for *Candida spp*. The highest percentage of *Candida spp* were isolated from oral swabs (60%), followed by peritoneal dialysate (34%), urine (24%), sputum (23%) and the lowest percentage was from pus (10%).

As shown in table (2), by the use of API 20 C AUX, the highest percentage of *Candida spp* isolated was *C albicans* {23 isolates (44.2%)}, followed by *C tropicalis* {13(25%)}, then *C glabrata* {6 (11.5%)}. One (1.9%) of each of *C krusei*, *C stellatoidea*, and *C kefir* was isolated. *C albicans* was the most frequently *Candida spp* isolated from every specimen type.

The intergenic spacer region was successfully amplified from all *Candida* isolates tested giving amplification product 510-871 bp. As shown in (Fig 1), after digestion of PCR product with *MspI*, 3 fragment were given for *C guilliermondii*, two fragments for *C.albicans*, *C krusei*, *C glabrata*,

C tropicalis and *C stellatoidea*, and no effect on *C parapslosis* amplicon.

In all *Candida* isolates, identification by RF PCR shows 86.5% agreement with API. RF PCR shows 100 % agreement with API identification in case of *C tropicalis*, *C*

glabrata, *C krusei* and *C guilliermondii*. In case of *C albicans*, 96% (22 out of 23) agreement of RF PCR is shown in comparison to API. RF PCR fails to identify isolates of *C kefyri*, *C lusitaniae* and *C parapslosis* to species level.

Table (1) shows specimens and number and percentage of positive sample

Specimen	Number of samples	Number of samples positive for yeast	% of samples positive for yeast
Blood	(40)	8	20%
Peritoneal dialysate	(35)	12	34%
Oral swab	(30)	18	60%
Sputum	(30)	7	23%
Urine	(25)	6	24%
Pus	(10)	1	10%
Total	(170)	52	30.6%

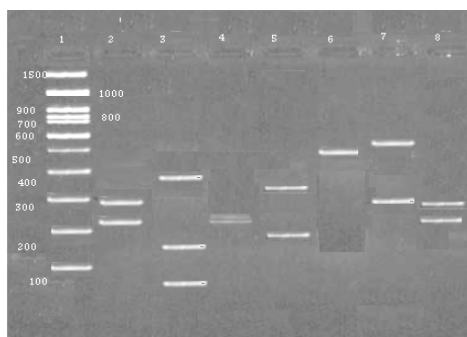


Fig (1): Gel electrophoresis after digestion of PCR product with *MspI* (RF-PCR). Lane1-8 Molecular weight marker, *C albicans*, *C guilliermondii*, *C krusei*, *C tropicalis*, *C parapslosis*, *C glabrata* and *C stellatoidea*

Table (2) shows number and percentage of *Candida spp* isolated from the samples identified by API 20C AUX

<i>Candida spp</i>	Blood	Peritoneal dialysate	Oral swab	Sputum	Urine	Pus	Total
<i>C albicans</i>	3 (37.5%)	4 (33.3%)	7 (38.9%)	4 (57.1%)	4 (66.7%)	1 (100%)	23 (44.2%)
<i>C tropicalis</i>	1 (12.5%)	3(25%)	5 (27.8%)	3(42.9%)	1(16.7%)	- (0%)	13(25%)
<i>C glabrata</i>	2 (25%)	1 (8.3%)	3 (16.7%)	- (0%)	- (0%)	- (0%)	6 (11.5%)
<i>C kefyri</i>	- (0%)	1 (8.3%)	- (0%)	- (0%)	- (0%)	- (0%)	1(1.9%)
<i>C parapslosis</i>	- (0%)	2 (16.7%)	1(5.6%)	- (0%)	- (0%)	- (0%)	3 (5.8%)
<i>C lusitaniae</i>	1 (12.5%)	- (0%)	1(5.6%)	- (0%)	- (0%)	- (0%)	2 (3.8%)
<i>C guilliermondii</i>	- (0%)	1 (8.3%)	- (0%)	- (0%)	1(16.7%)	- (0%)	2 (3.8%)
<i>C krusei</i>	1 (12.5%)	- (0%)	- (0%)	- (0%)	- (0%)	- (0%)	1(1.9%)
<i>C stellatoidea</i>	- (0%)	- (0%)	1 (5.6%)	- (0%)	- (0%)	- (0%)	1(1.9%)
Total	8 (15.4%)	12 (23.1%)	18 (34.6%)	7 (13.5%)	6 (11.5%)	1 (1.9%)	52(100%)

Table (3): Shows comparison of identification of isolated *Candida spp* by API versus RF-PC

Species	API	RF-PCR	Agreement
<i>C albicans</i>	23	22	96%
<i>C tropicalis</i>	13	13	100%
<i>C glabrata</i>	6	6	100%
<i>C kefyri</i>	2	0	0%
<i>C parapslosis</i>	3	0	0%
<i>C lusitaniae</i>	2	0	0%
<i>C guilliermondii</i>	2	2	100%
<i>C krusei</i>	1	1	100%
<i>C stellatoidea*</i>	1	1	100%
Total	52	45	86.5%

**C stellatoidea* gives the same bands as *C albicans* after RF PCR

4. Discussion:

The frequency of invasive mycoses due to opportunistic fungal pathogens has increased significantly over the past two decades. ^(13-16,18) *Candida species* are the most common opportunistic fungal pathogens in humans ^(17,19).

As shown in table (1), 52 (30.6%) out of 170 specimen, collected from 120 immunocompromized patients, were positive for *Candida spp*. The highest percentage of *Candida spp* were isolated from oral swabs (60%), followed by peritoneal dialysate (34%), then urine (24%), sputum (23%) and the lowest percentage was from pus (10%).

These infections could be associated with excessive morbidity and mortality and is directly related to increasing patient populations at risk for the development of serious fungal infections, which includes individuals undergoing solid-organ transplantation, blood and bone marrow transplantation, and major surgery and those with AIDS, neoplastic disease, immunosuppressive therapy, advanced age, and premature birth ⁽²⁰⁾.

Candidiasis is the most common oral fungal infection in humans ⁽²¹⁾. In recent years, although *C. albicans* remains the most common cause of oral candidiasis, the proportion of cases in which *C. glabrata* has been isolated is increasing. Redding *et al.* reported the emergence of *C. glabrata* infection in individuals receiving radiation for head and neck cancer ⁽²²⁻²⁴⁾.

As shown in table (2), by the use of API 20 C AUX, the highest percentage of *Candida spp* isolated was *C albicans* {23 isolates (44.2%)}. This is in agreement with other studies ^(17, 19, 25). Non-*C. albicans* species are emerging pathogens and can also colonize human mucocutaneous surfaces ⁽²⁶⁾. Consequently, they are also isolated in the setting of candidiasis, albeit at a lower frequency. This was proved by our study as non *C albicans* species isolated were as follows: *C tropicalis*(25%), *C glabrata*(11.5%), *C parapsilosis*(5.8%), *C lusitanae*(3.8%), *C guilliermondii*(3.8%), *C stellatoidea*(1.9%), *C kefir* and *C krusei*(1.9%). Thus the most commonly isolated *Candida spp* include *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*. This is in agreement with previous studies ^(27,28).

Non *Candida albicans* are emerging opportunistic pathogen and in the same time the use of empiric antifungal drug can lead to unfavorable outcome as some non *Candida albicans spp* are intrinsically resistant to some drugs so a rapid method of identification will be very helpful in management and epidemiology of fungal infection especially in immunocompromised patients.

Molecular techniques are targeted to detect *Candida species* in a short period of time, with high sensitivity and specificity. Restriction enzymes are endonucleases that cleave DNA in recognition sites on the DNA. The recognition site consists of a specific sequence of nucleotides in the DNA duplex, typically 4-8 bp. Experiment with restriction enzyme is simple, relatively inexpensive and their result is reproducible. These enzymes have vast application in the molecular biology especially for the diagnostic purposes ^(10,29). So we tried identification of *Candida spp* using RF PCR. The primer used gives an amplicon (510-871 bp) from all *Candida* isolates tested. This result was in agreement with previous studies ^(10,29).

In this study, after subjecting amplicon to the enzyme *MspI*, gel electrophoresis separates bands specific for some species identifying them to the species level. Comparing RF-PCR with API, Identification by RF PCR shows 86.5% agreement with API in all *Candida* isolates. Identification by RF-PCR shows 100 % agreement with API identification in case of *C tropicalis*, *C glabrata* and *C guilliermondii*. 22 out of 23 *C albicans* isolates (96%) agreement in identification by both methods was shown. Thus, it can identify the most commonly isolated *candida spp* to the species level. RF PCR fails to identify isolates of *C stellatoidea*, *C lusitanae*, and *C parapsilosis* to species level.

The study of Ayatollahi *et al* applying the same method gave 100 % agreement with phenotypic methods. His experiment gave two bands for each of *C. albicans*, *C. tropicalis*, *C. krusei* and *C. glabrata* and three bands for *C. guilliermondii* ⁽³⁰⁾. We give identical results as to all isolates of *C tropicalis*, *C glabrata*, *C guilliermondii* and *C krusei*. But only (22 isolates out of 23) 96 % agreement with *Candida spp* as one isolates API identified as *Candida albicans* failed to give specific bands by RF PCR. This isolate may have mutation in the restriction site of restriction enzyme *MspI*. Although it is a rare possibility but it can occur. It may be also falsely identified as *Candida albicans* by the phenotypic method.

In this study, *C stellatoidea* isolate gives two bands identical to the bands given for *C albicans*. Isolates of *C. stellatoidea* have almost always been distinguished from *C. albicans* by their lack of sucrose assimilation. This isolate may be sucrose-negative *C. albicans* according to Kwon-Chung *et al.* ⁽³¹⁾ who demonstrated that the isolates identified as *C. stellatoidea* on the bases of carbon assimilation tests can be either type I or type 11, depending on their electrophoretic karyotypes. The isolates designated as type II were considered to be sucrose-negative *C. albicans*, while type I isolates were considered to be

C. stellatoidea.⁽³²⁾ Out of 681, only 1 isolate was identified as type I *C. stellatoidea* using contour-clamped homogeneous electric field electrophoresis as described by Chu *et al.*⁽³³⁾ The remaining isolates were found to be *C. albicans* (98.9%) and other yeasts (1.1%). Identification of *C. stellatoidea* by PCR RFLP was done by using the restriction enzymes *BfaI* and *DdeI* to restrict the amplicon by Williams *et al.*⁽³⁴⁾

This study concludes that *Candida albicans* is still the most important candida spp affecting immunocompromised patients and Non *Candida albicans spp* are emerging important pathogens. It also concludes that RF-PCR using the restriction enzyme *MpsI* is a good rapid identification method that identifies the most important *Candida spp* isolated from immunocompromised patients and recommends further studies to develop new methods using different restriction enzymes to increase the range of identified *candida spp*.

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