



Differentiation between *C. glabrata* and *C. parapsilosis* Isolated from Children with Diarrhea by PCR Amplification of the ITS1-5.8S-ITS2 rDNA



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Abstract

Candida glabrata and *Candida parapsilosis* are most important non-albicans species which are considered pathogenic to human and isolated from children with diarrhea. The purpose of the study is the identification and differentiation between *Candida glabrata* and *Candida parapsilosis* among children with diarrhea by PCR and determination relation of this species with gender, age group and resident of children. Hundred stool samples were collected from children with diarrhea seeking health care at AL-Batool Hospital in Baqubah city. The Stool cultured on sabouraud dextrose agar, subcultured on Chromogenic agar and PCR methods identification. In the present study, the isolated were identified which 14/64 (21.9%) isolates were *C. glabrata*, while 11/64 (17.2%) isolate *C. parapsilosis* and 39/64 (60.9%) isolates were other *Candida* spp, the results showed there is a significant relationship between *C. glabrata* and *C. parapsilosis* with the gender of children. *Candida glabrata* and *Candida parapsilosis* have a close color between of them in Chromogenic agar but when PCR amplifying the ITS1-5.8S-ITS2rDNA region produced different size base pair that makes it easy differentiation between them.

Keywords: *C. glabrata*; *C. parapsilosis*; Diarrhea; ITS1-5.8S-ITS2rDNA; PCR

Introduction

Candida glabrata and *Candida parapsilosis*, the two genus belongs to the kingdom Fungi, division Eumycota (true fungi), which relates to class Deuteromycetes (Fungi imperfection) and the family Saccharomycetaceae (budding yeast) containing different genera of yeast, the genes of *Candida* which is one of the most common yeasts.

Candida glabrata constituting number two in greatest virulent yeast following *C. albicans*. *C. glabrata* does a haploid yeast, non-dimorphic, don't production pseudohyphae at 37 °C. *C. glabrata* to be more in people with low immunity state, *C. glabrata* to be a very opportunistic of the both urinary, genital organs and the blood circulating through the body of a person, while *C. parapsilosis* is a diploid yeast, dimorphic and don't formation true hipe *C. glabrata*. That is particularly widespread in people living with HIV and the aged [1]. In most surveys conducted in the US and Europe, *C. glabrata* is the second most common *Candida* species leading to invasive fungal infections [2]. By comparison, in Asian - Pacific countries and Latin America, *C. tropicalis* and *C. parapsilosis* are the second and third most common *Candida* spp., respectively [3].

Recently, there is a decrease in frequency of *C. albicans* and an increase in *C. tropicalis* and *C. parapsilosis*, while the frequency of *C. glabrata* and *C. krusei* has remained unchanged. *C. glabrata* is also prevalent in the elderly, and frequency of *C. parapsilosis* decreases with age [2].

Candida is naturally present in humans and it does not cause any health problems in the host, but it works to destroy harmful bacteria. In individual who has good health, the proportion of *Candida* yeast is low due to the effectiveness of the immune system in addition to the presence of Probiotics bacteria such as Lactobacilli, however if the rate of these bacteria is low due to the defect of the immune system in the body or the use of antibiotic drugs, which leads to excessive growth of fungus, special *Candida* spp. and then change from the yeast to mycelium and then begin to penetrate the tissue host and causes candidiasis [4].

Yeast cells are approximately 2-10µm in the largest dimension, round to oval, and they are reproduced by budding. *C. albicans* is the lager yeast at 4-6 x 6-10µm, whereas *C. glabrata* and *C. parapsilosis* is the smallest at 1-4 x 2-9µm and 2-4 x 2-9µm, respectively [5].

Candida-associated with diarrhea in hospitalized patients, when there is a prior use of antibiotics. The yeast overgrowth is presumed to be antibiotics disruption of the normal flora. There is a mystery between Candida spp. Infection and the mechanism that causes diarrhea. It has been shown that Candida spp. can block lactase activity intestine that leads to lactose intolerance [6].

The Symptoms of Candida-associated diarrhea comprise lengthened diarrhea with abdominal ache, and suffer from colic but absence blood or mucus, nausea, vomiting, fever. The research and report showed that the clinical signs of the diarrhea were reduced and that coincides with the decrease fungus in the stool and the use of special fungicides [7].

Several molecular techniques have emerged for detection of Candida species which are based on amplification or hybridization of target nucleic acids, these are: Polymerase Chain Reaction (PCR) [8]. The genome of the fungi contain rRNA gene which is consisting of three subunits, these including small subunit (18S), 5.8S gene, and a large subunit (25S) gene, they are separated by the Internal Transcribed Spacer-1 (ITS1), and ITS2 regions. The ITS zone is a segment of non-functional RNA sequence which is located between rRNAs on a common precursor transcript, and it is usually used for identification of medically important fungi [8,9].

Material and Methods

Collection of samples

Hundred fecal specimens were collected from children under three years in cups. The patient's name, age, gender were fixed in each cup and diagnosed immediately.

Culturing of samples

Sabouraud dextrose agar (SDA) was your utilized medium for the main isolation of Candida spp., it allows the growth of Candida and represses the expansion of many bacteria because of the low pH. Therefore; SDA was cooled down to 45-50 °C then chloramphenicol antibiotic (250mg/liter) it was added then poured into sterile Petri dishes. SDA medium was inoculated with a small portion of the stool specimen with a sterile loop. The dishes remained incubated at 37 °C for 48hrs. And covered the plates by using the parafilm to preserve the culture and prevent contamination when saved in the refrigerator [10].

Chromogenic agar candida (CAC)

Chrome agar Candida pure isolates of Candida were resuscitated by inoculating a loop full of culture from Sabouraud Dextrose Agar into 10ml sterile broth that incubated at 37 °C for 3 days. After 72 hours of incubation, cultures were inoculated on the surface of the Chrome agar media by streaking a loop full of culture and incubated at 37 °C for 72-96 hours with the plate facing upright. After 72-96 hours of incubation, all plates were removed from the incubator and the results were recorded. Candida colonies were initially identified by colonial color when compared with standard color photographs supplied by the manufacturer and also presented [11].

Molecular identification

DNA extraction: DNA was extracted from Candida spp. using Wizard Genomic DNA purification kit (Promega, USA) according to the protocol stated by the kit manufacturer.

DNA extraction procedure occurred by the following steps: Adding one ml of culture yeast for a period of twenty hours in the broth yeast extract-peptone-dextrose medium to a micro centrifuge tube. Centrifuging at 15,000 × g for two minutes to pellet the cells. Eliminate the supernatant. Resuspend the cells completely in 293µl of 50mM EDTA. Add 7.5µl of lysis buffer, then carefully mix it with a pipette. 5. Placing the specimen in the water bath at 37°C for one hour and cool to room temperature. Centrifuge at 15,000 × g for two minutes, after that, eliminate the supernatant. Adding Nuclei Lysis Solution amount 300µl to the cell pellet and then carefully mixing it with a pipette. Adding Protein Precipitation Solution amount 100µl and vortex quickly. Putting the specimen on the ice at five minutes. Centrifuging at 15,000 × g at three minutes. Preparing new microcentrifuge tube containing 300µl of the isopropanol then transfer supernatant containing DNA to it. 12. Inversing mixing continuously till the thread-similar strands of DNA from a noticeable piece. Centrifuging at 15,000× g for two minutes. 14. Emptying the supernatant and drain the micro centrifuge tube on new absorptive paper. Add 300µl of the 70% ethanol and invert the micro centrifuge tube sometimes to wash the DNA. Centrifuging at 15,000× g at two minutes and aspiration whole of the ethanol. 16. Draining the tube on clean absorbent paper and leave the pellet to air-dry for 12 minutes. Adding 50µl of DNA Rehydration Solution. 18. Adding 1.5µl of RNase Solution to the purified DNA sample. Vortex the sample for 1second. Centrifuge at 5 seconds to collect the liquid and incubate at 37°C for 15 minutes. Rehydrating the DNA by incubating the solution one night at 4°C. 20. Storing the DNA at 2–8°C.

Materials used for thermal cycling

Universal primers ITS1 (TCC GTA GGTGAA CCT GCG G) and ITS4 (TCC TCC GCT TATTGA TAT GC) were used to amplify the ITS1-5.8S-ITS2 fragment of fungal [12,13]. We're synthesized by (Bioneer Co., USA) Optimization of PCR was accomplished after several trials. Thus the following mixture was adopted amplification reactions were produced in the 25µl final volume containing 12.5µl Go Taq® master mix (Promega, USA), 5µl of the primers and 5µl DNA template and complete the volume by Nuclease-Free Water. There are two programs thermal controller use in this study, Program for amplifying the ITS1-5.8S-ITS2 for Candida spp. to the identification of Candida spp. An initial denaturation step at 94 °C for five minutes was followed by twenty-five cycles of denaturation at 95°C for thirty seconds, annealing at 56 °C for fifty-four seconds, and extension at 72 °C for one minute, with a final extension step of 72 °C for Seven minutes [13] Program for amplify the 25S for Candida albicans to identification Candida albicans genotypes, following thermal cycling: 94 °C for three minutes followed by thirty cycles of denaturation at 94 °C for one minute, annealing at 55 °C for one

minute, extension at 72 °C for two and a half minutes and a 72 °C for ten minutes for a final extension following the last cycle [14].

Electrophoretic

Electrophoretic separation of DNA fragments was done according to the method described by Fankhauser [15]. Equipment: Ultra-clean glassware, 1L beaker, 1L graduated; 250ml beakers cylinder, 100ml graduated cylinder horizontal, gel tray; electrophoresis unit, power supply, well comb, micropipettes, UV transilluminator. Supplies: latex gloves, 1X TBE buffer, agarose 1.5%, 5v/cm 2hr., micro centrifuge tubes [15].

Results

Cultural characteristics

The results of specimens by the culture of SDA showed that 64 isolates of *Candida* infection. Morphological culture on SDA, colonies of *Candida* spp. colonies on sabouraud dextrose agar were white to creamy, round, soft, and smooth to wrinkled with a characteristic yeast odor; examination of *Candida* spp. isolates showed spherical to oval cells with a presence of budding and was larger than bacterial cells, as it appears in as is shown in (Figure 1).

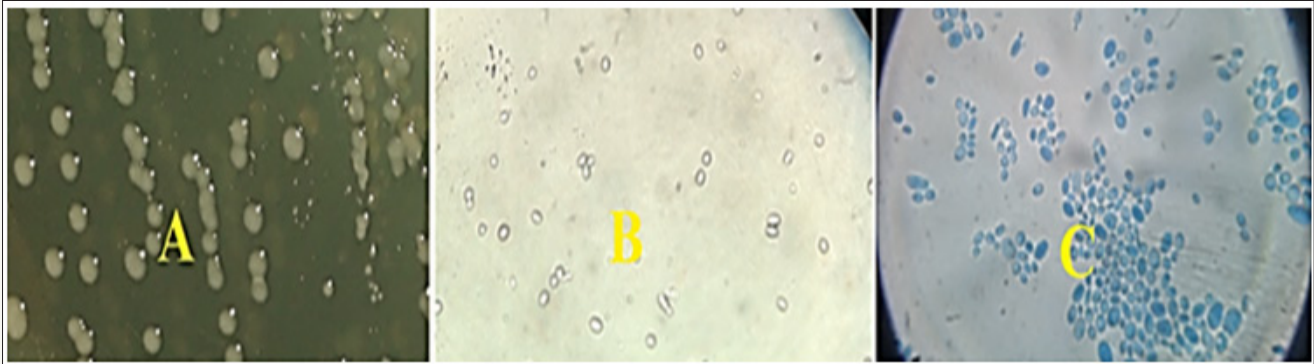


Figure 1A: *Candida* spp. cultured on SDA at 37 °C for 48 hrs,
B: *Candida* spp without stained (40X),
C: *Candida* spp stained with Lactophenol cotton blue (40X).

Chromogenic agar candida (CAC)

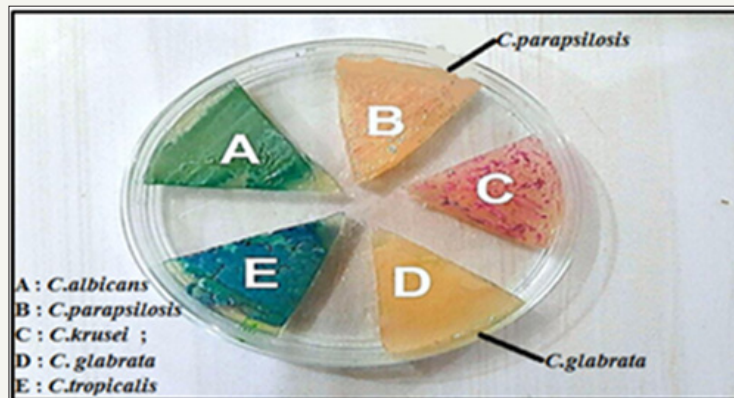


Figure 2: Colonies of *Candida* spp. cultured on chromogenic agar *Candida* at 37 °C for 48 hrs, appeared easy distinction colors between *C. albicans*, *C. krusei*, *C. tropicalis*, but there are Colors are close to *C. glabrata* and *C. parapsilosis*.

Where several colours emerged from the growth of *Candida* in that medium, were such as with the company protocol, greenish colonies of *C. albicans*, blue colonies of *C. tropicalis*, Purple- Pink colored colonies of *C. krusei*, *C. glabrata* produced cream to white and *C. parapsilosis* produced pinkish to white, as it appears in (Figure 2). In the present study, there were differences between *Candida* spp., 14/64 (21.9%) of the total isolates were identified *Candida* as *C. glabrata*, while 11/64(17.2%) isolate *C. parapsilosis* and 39/64 (60.9%) isolates were other *Candida* spp. *C. albicans*, *C. krusei* and *C. tropicalis*.

Molecular identification

Fungus-specific universal primer pairs (ITS1 and ITS4) were able to successfully amplify the ITS1-5.8S-ITS2 region of all fungi tested, providing a single PCR product of approximately (510-870 bp) [13,16]. The 64 sample were molecularly identified according to ITS1-ITS4 sizes polymer chain reaction products for *Candida* spp. show that *C. glabrata* (871bp), while *C. parapsilosis* (520bp), as it appears in (Figure 3).

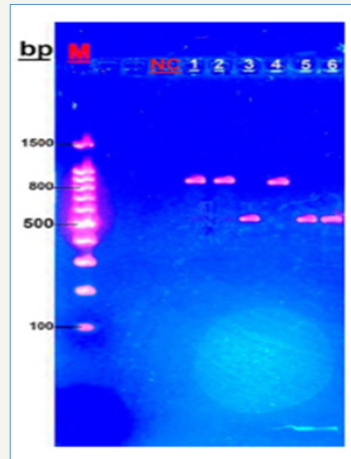


Figure 3: Agarose gel electrophoresis of Candida DNA products generated through primer ITS1 and ITS4, stained with ethidium bromide Lane M: Molecular marker (100bp); lane NC: negative control; lane, 2,4: *C.glabrata*; lanes 3,5,6: *C.parapsilosis*.

In this study, the results showed there are significantly ($P < 0.05$) relationship between *C. glabrata* and *C. parapsilosis* with children gender as shown in (Table 1). The results also showed there are insignificantly relationship between *C. glabrata* and *C. parapsilosis* with children and age group as shown in (Table 2). They also insignificantly relationship between *glabrata* and *C. parapsilosis* with children and mode of feeding as shown in (Table 3).

Table 1: Candida spp the infection rate among patients according to gender.

Candida spp.	Gender		Total	
	Male	Female		
<i>C. glabrata</i>	NO	6	8	14
	%	42.86%	30.77%	100.00%
<i>C. parapsilosis</i>	NO	9	2	11
	%	23.68%	7.69%	100.00%
Total	NO	14	10	25
	%	100.00%	100.00%	100.00%

P-value= 0.0483 sig. ($P < 0.05$)

Table 2: Candida spp. the infection rate among patients according to age.

Candida spp.		Age (month)					Total
		≥ 2	3-5	6-11	12-23	24-36	
<i>C. glabrata</i>	No	0	4	7	2	1	14
	%	0.00%	28.60%	50.00%	14.30%	7.10%	100.00%
<i>C. parapsilosis</i>	No	3	3	2	2	1	11
	%	30.00%	30.00%	20.00%	10.00%	10.00%	100.00%

p-value =0.227 non sig. $p > 0.05$.

Table 3: Candida spp. the infection rate among patients according to feeding.

Candida spp.		Mode of Feeding				Total
		Breast Feeding	Bottle Feeding	Mix Feeding	Raw Food	
<i>C. glabrata</i>	No	2	9	2	1	14
	%	14.30%	64.30%	14.30%	7.10%	100.00%
<i>C. parapsilosis</i>	No	0	8	2	1	11
	%	0.00%	72.70%	18.20%	9.10%	100.00%

p-value= 0.631 non sig. ($p > 0.05$)

Discussion

Intesar and Ahmed in Iraq, showed in their study from 23 isolates of *Candida* spp. were 3 (9.4%) of both isolate of *C. parapsilosis* and *C. glabrata* that isolated from children with diarrhea [17]. A study by Zang L. et al. in China showed that *C. glabrata* was (20.3%) and *C. tropicalis* was (8.8%) [18]. In a study for identifying *Candida* spp. on chrome agar found 45 *C. glabrata* produced variously colored colonies on this medium, 29 White and 16 Lavender [19]. (2008) noted that 51 *C. glabrata* produced variously colored colonies on this medium, i.e., 37.8% maroon, 31.1% beige, 28.9% yellow, and 2.2% violet [20]. The ITS1 and ITS4 universal primer pairs were able to successfully amplify the ITS1-5.8S-ITS2 region of all fungi tested, providing a single PCR product of approximately (510-870bp) [13,16]. Our study agreed with Mirhendi e and Wahyuningsih, that found molecular identified according to ITS1-ITS4 sizes polymer chain reaction products for *Candida* spp. show that *C. glabrata* (871bp) and *C. parapsilosis* (520bp) [21].

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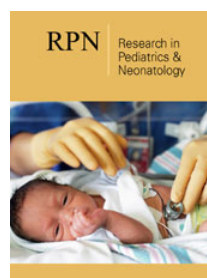
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