Molecular& Antifungal Suceptibility Identification of *Candida albicans* Isolated from Samples of GIT Children with Diarrhea in Diyala Province Iraq

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Abstract

Diarrheal diseases in children are a major public health concern in developing countries. especially species of *Candida*, responsible for causing candidiasis infection(diarrehea), require fast and accurate identification, The correct identification of *Candida* species is of great importance, as it presents prognostic and therapeutical significance, allowing an early and appropriate antifungical therapy. This study was conducted to investigate the incidence of *Candida albicans* associated with diarrhea in children

This study was included isolates of *Candida* spp. from stool of 100 patients samples with diarrhea and 100 without diarrhea evaluated in AL-Batool Teaching Hospital by phenotypic methods , antifungal susceptibility of *C. albicans* and determination of the *C.albicans* genotypes by Nested PCR, discriminating *C. albicans* from the other *Candida* species. The tests used for phenotypic analysis were culture in SDA and CHROMAgar™ Candida, Phenotypic tests showed green colonies in chromogenic medium, The antifungal susceptibility results of 43 *C.albicans* isolates present that all of *Candida* isolates tested were susceptible to Amphotericin B and fluconazole (100%), while no resistance was observed in *C.albicans* toCaspofungin and Micafungin.

Finally, only one isolate was resistant to flucytosine . Genomic DNAs of all *C. albicans* isolates were amplified by PCR to detect their genotypes using. polymerase chian reaction amplification shows four genotypes(A,B,Cand D).A-genotype showed 4 subtypes and most common subgenotypes(1,3,4 and 5).

Kew word: *C.albicans, Nested PCR, Antifungal suceptibility genotype identification*

Introduction

Candida species is the genus comprises about 150 yeast species, the genus is composed of a heterogeneous group of organisms, and more than 17 different *Candida* species are known to be etiological agents of human infection; however, more than 90 % of invasive infections are caused by *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis* and *Candida krusei* [14].

Candida is a part of normal flora of the human body colonizing various anatomical site like oral cavity, digestive tract, vagina and skin[19]. In cases where there is host debilitation, or where there is a change in the local environment promoting *Candida* overgrowth, this led to *candida* infection referred to as candidiasis) [7].

C albicans is the most common yeast species isolated from human feces, being identified in 65% of stool samples from healthy adults.Nevertheless, several reports have suggested that it may cause diarrhea. [3].

Diarrhea is a major cause of child morbidity and mortality in socio-economically developing countries. More than one million episodes of diarrhea occur every year among children under five years of age causing approximately 2.5million deaths[12],[16].

Molecular method for direct identification of *Candida* species has proven to be an accurate and rapid method for detection of candidal infections [16],[23] .

Nested polymerase chain reaction (Nested PCR) is a modification of polymerase chain reaction intended

to reduce non-specific binding in products due to the amplification of unexpected primer binding sites[5].

Material and Methods

Isolation and Identification of *Candida spp*.

Stool sample were collected from diarrheal children under 5 years old came to the microbiology laboratory in AL-Batool Teaching Hospital, general stool examination and Stool culturing during two months period (from 1st September 2018 to end November 2018).

All the collected samples were cultured directly, on Sabouraud dextrose agar (SDA) containing Chloramphenicol. Then incubated for 48 hrs. And the only positive samples were cultured on CHROM Agar (CAC),[13].

Antifungal susceptibility test

Antifungal susceptibility test was done according to (Vitek 2 Compact), The YST identification card is based on established biochemical methods and newly developed substrates [2]. Final identification results are presented in approximately 14-18 hours.

The VITEK 2 cards containing serial twofold dilutions of Amphotericin B, Fluconazole, Flucytosine, and Voriconazole,Caspofungin,Micafungin were provided by the manufacture.

Molecular Methods – based PCR.

DNA extraction.

Genomic DNAs were extracted from *C.albicans* isolates, using a QIAamp DNA Mini Kit according to the manufacturer's (Qiagen DNA extraction kit) modified protocol. The purified DNA was stored at 20 ºC until further analysis. Quantus Florometer was used to detect the concentration of extracted DNA in order to detect the purity of samples for downstream applications.

For 1 µl of DNA, 199 µl of diluted QuantyFlour Dye was mixed. After 5min incubation at room temperature, DNA concentration values were detected, DNA concentration appeared at rate from 2-20 (ng/µl).

Candida albicans Identification by Nested PCR

In order to identify *Candida albicans* genotypes and subgenotypes on the basis of 25S rDNA, Two sets of primers were used to increase these sensitivity and specificity of the assay, *C.albicans* was classified into five genotypes: A, B, C, D, and E [4],[15].

Identification of Candida albicans genotypes

The specific primer pairs used to detect the 25S rRNA were CA-INT-L (ATA AGG GAA GTC GGC AAA ATA CCG TAA) and CAINT-R (CCT TGG CTG TGG TTT CGC TAG ATA GTA GAT) [12].

Identification of Candida albicans subgenotypes

The primer set ASDcF : 5`-TGATGAACCACATGTGCTACAAAG-3`

And pCSCR: 5`-CGCCTCTATTGGTCGAGCAGTAGTC-3`

Is referred to as P-II in this study used for identify subgenotype of *C.albicans* [4].

all primers were synthesized by (Bioneer Co., USA). These primers were suspended in Nuclease-Free water to be 100 pmol/ µl as definitive concentration, as recommended by company protocol, put in freezer until time of use.

Statistical Analysis.

All data were statistically analyzed depending on SPSS (Statistical Package for Social Science) version 18 (2009).Chi-square and Yates's correction was used to compare the variable in this study. Statistical results were considered significant when being under or equal to the 0.05.

Results and Discussion.

Isolation and Identification of *Candida* spp.

Morphological culture on SDA, colonies of *C. albicans* on sabouraud dextrose agar were white to creamy, round, soft, and smooth to wrinkled with a characteristic yeast odor as presented in figure 1) .[16].

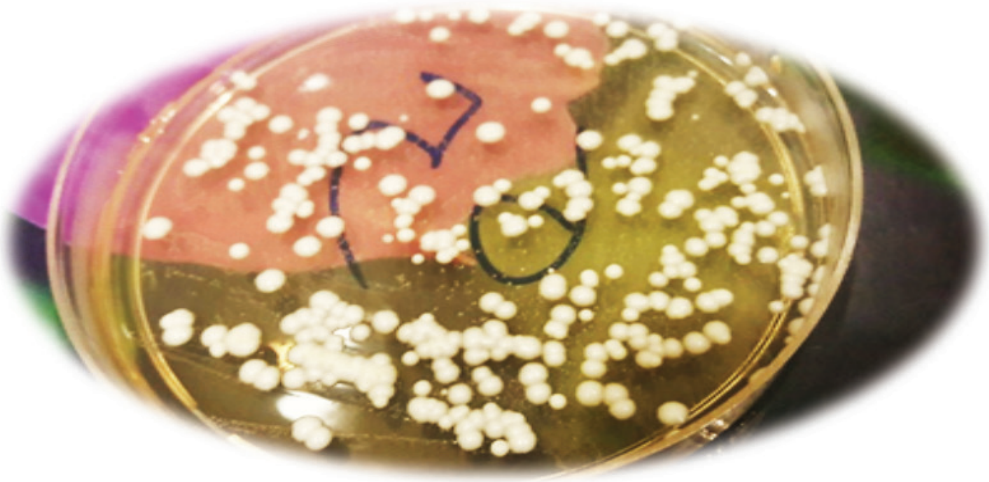

Figure 1 : Colonies of *Candida* spp. (Pure Culture) on SDA at 37ºC for 48 hrs

The results of this study are in agreements with [23], in thi-Qar that found the total rate of *candida* infection was (64.4%) of 264 examined stool specimens at Al-Zahraa health center,Suq Al-Shuyukh district. In another study from Kurdistan,Iraq ,600 stool speciemen from children in different age(<2-12years) were examined not only for *Candida* spp. but also for enteropathogenic bacteria, parasites, and viruses; *Candida* spp. could be detected in 1.16% of diarrhea cases[19].

The present study indicate that difference between *Candida* spp., 43 (64.71%) of the total isolated were identified as *C. albicans*, 24 (35.82%) isolates were identified as non-*albicans*., of which *Candida tropicalis* 8 (11.94%), *Candida glabrata* 6 (8.95%), *Candida krusei* 5 (7.46%), *Candida parapsilosis* 5 (7.46%) as shown in table 1.

Table 1 *Candida* spp. isolated from children stool.

Candida species	No. of isolates	Percentage %
Candida albicans	43	64.17%
Candida tropicalis	8	11.9%
Candida glabrata	6	8.9%
Candida krusei	5	7.4%
Candida parapsilosis	5	7.4%
Total	67	100%

Candida species' well growth on chromogenic medium in this work supported the fact about this medium has a good performance, less time consuming, and sensitive in detection of *Candida* species[22]. These results also confirmed that chromogenic agar had a major role in the classification of *Candida* spp. into *C. albicans* and non-*albicans* [3],[15].

Antifungal susceptibility test.

In this study, Vitek 2 YST was evaluated using 43 isolates of commonly observed species of Candida (*C.albicans*). The result generated as the Vitek 2 AST-YSO7 Card evaluated the sensitivity and resistance of Amphotericin B, Fluconazole, Micafungin, Caspofungin, Voriconazole, and Flucytosine.

The antifungal susceptibility results of 43 *Candida. albicans* isolates present that All of *Candida* isolates tested were susceptible to AMB and fluconazole (100%). The resistance was high in *C. albicans* against

AMB(34.88 %) and then against fluconazole and Voricanozole (32.55% & 25.58%) respectively, while no resistance was observed in *C.albicans* against Caspofungin and Micafungin. Finally, only one isolate of each of the following isolates were resistant to flucytosine in percentage (2.32%). as showed below in (figure.2).

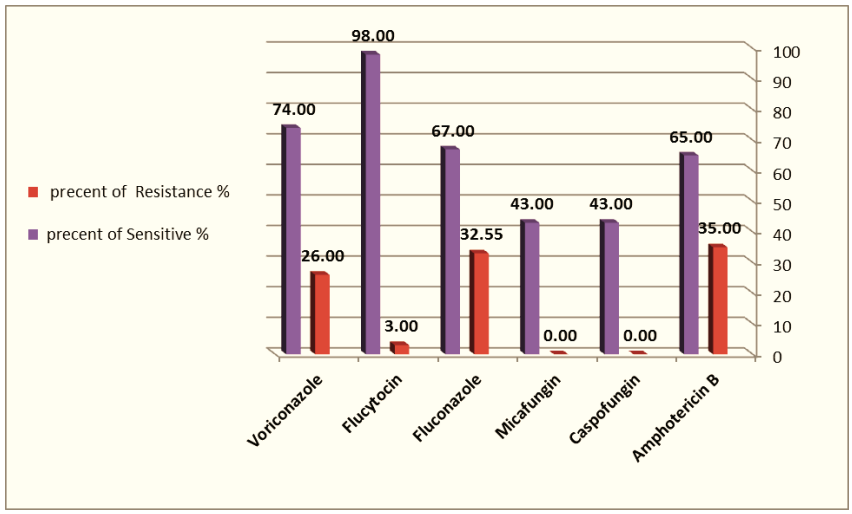

Figure 2: Percentage of *C.albicans* isolates according Sensitivity and Resistance to antifungal agents.

These results were disagreement with (Rehab *et al* ;2011) who found that non of *Candida* isolates were resistant to AMB and only few isolates of *C.albicans* were resistant to FCN

Moreover, This result was agreement with [21] in Diyala who found that the antifungal drugs sensitivity test against *C.albicans* showed that all *C.albicans* isolates (100 %) resistant.

Molecular Method for Detection of *Candida albicans*.

Genomic DNAs of all *C. albicans* isolates were amplified by PCR to detect their genotypes using primer pairs (CA-INT-L and CA-INT-R) by targeting the gene 25S rDNA. polymerase chian reaction amplification shows four genotype(A,B,C,D) as its shown in figure[3].

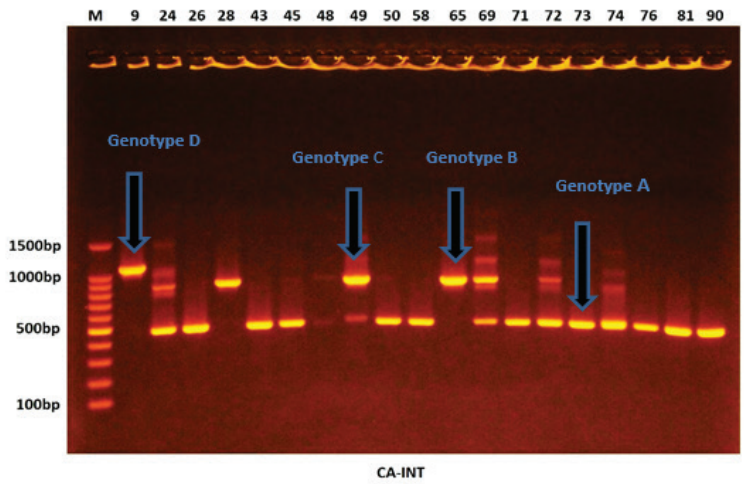

Figure 3:Agarose gel electrophoresis of *C. albicans* genotypes by PCR targeting 25S rDNA on 1% A garose gel, 5 volt/cm for 1.5 hr, stained with ethidium bromide dye. Genomic DNA products generated through The primer pairs CA-INT-L and CA-INT-R, of 450 bp for genotype A in lanes: (26,43,45,50,58, 71,73,76,81,90). 840 bp for genotype B in lanes: (28,65). Both 450 bp and 840 bp for genotype C in lanes: (24,48,49,72,74).1040 bp for genotype D in lanes:(9).

For determination of *C. albicans* on the basis of 25S rDNA, primers CA-INT-L and CA-INT-R that span the site of the transposable intron in the 25S rDNA [15] were used in this study. This method has been confirmed as a specific and reproducible method of genotype analysis of *C. albicans* [8]. PCR targeting 25S rDNA allows *C. albicans* to be grouped into five genotypes A, B, C, D and E[8],[10],[13],[15].

In this study; gel electrophoresis profiles defined DNA products as following: 450bp for genotype A, 840bp for genotype B, both 450&840bp for genotype C,1040 pb for genotype D,the study considered this isolate to belong to a new genotype ,None of PCR product in this study were genotype E 1080bp.

In addition to above, the most prevalent *C. albicans* genotype among Gastrointestinal candidiasis isolates on the basis of amplification of 25S rDNA was genotype A (52.6%), followed by genotype C (31.5%),and B (10.5%), and D (5.2%),this result agree with [10],[21] in Diyala that found the C. *albicans* genotype A is the most frequent genotype in patients but disagree there result that genotype A Followed by genotype B and C respectively.

Identification of Candida albicans subgenotypes

Candida albicans- specific primer pairs (ASDc-F and ASDc-R) were able to successfully amplify the region of the 25S rDNA gene. Polymerase Chain Reaction amplification shows four subgenotypes(1,3,4,5) according product size from any of the genotype A,B,C,D *C.albicans* from 4 isolates ,as it shown in Figure-5.

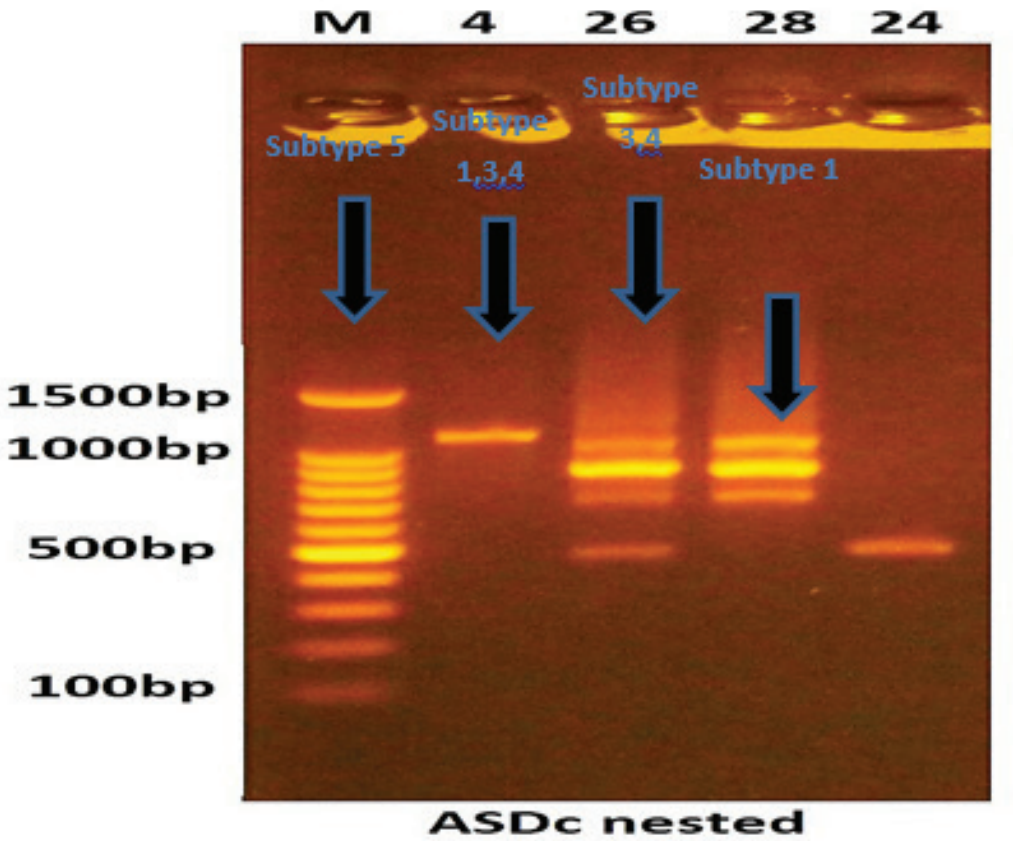

Figure 4: Agarose gel electrophoresis of *C. albicans* subgenotypes by PCR targeting 25S rDNA on 1% Agarose gel, 5 volt/cm for 1.5 hr, stained with ethidium bromide dye. Genomic DNA products generated through The primer pairs ASDc-F and ASDc-R, .

Another study in Turkey by[15]showed that genotype A (50.2%) was the most predominant genotype among invasive isolates annon-invasive isolates from sputum, throat, urine, and feces, followed by genotype C (31.9%), and genotype B (17.9%).

Total of 200 children less five years were included in this study. 67 (67%) children had positive Candidiasis. These highly significant differences between *Candida* spp., 43 (64.17%) were identified as *Candida albicans*, 24 (35.82%) isolated were identified as non-*albicans*.

In this study, we used Nested PCR that can specifically identify *C. albicans* at the species level, and we found 4 sub genotypes (1, 3,4and 5) based on PCR amplification generated by primer pair P-II (ASDc-F and ASDc-R) these genotypes were based on the size of product.[10],[11].

Result showed that single amplification product size 526bp as subtype 1 for the *C.albicans* genotype A,C, and single amplification product size 870bp as subtype 3 for the *C.albicans* genotype A,B,and amplification product size 1042 as subtype 4 for the *C.albicans* genotype A,B, and amplification product size 1214 as subtype 5 for the *C.albicans* genotype D.

Conclusions

From this study we conclude that about 67% of diarrheal cases were associated with Candidiasis.the antifungal test revealed that all of *C. albicans* isolates were susceptible to AMB and fluconazole while no resistant were observed against Caspofungin and Micafungin . polymerase chian reaction amplification shows four genotype(A,B,C,D).Agenotype showed 4 types and most common sub genotypes(1,3,4 and 5).

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Conflict of Interest: None to declare.

Ethical Clearance: All the strategies that we had followed were approved by the administration of our hospital and were carried out in accordance with who and the CDC guidelines

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