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Identification of *Candida* Species Using PCR/RFLP and Evaluation of ERG-11 Gene Expression in Neonatal and Pediatric ICUs

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Abstract

Infections caused by the wide diversity of *Candida* spp. stand for a critical health care problem in neonatal and pediatric intensive care units. The emergence of newly isolated strains that exhibit a resistance pattern has consequently led to a state of treatment failure. Hence, the accurate identification of *Candida* species as well as recognition of genotypic and phenotypic patterns of resistance is essential to resolve the dilemma of recurrent treatment failure. In this study, we aimed at genotypic and phenotypic characterization of clinically isolated *Candida* species by restriction fragment length polymorphism (RFLP) as well as screening of the identified species for expression of ERG-11 gene using RT- PCR technique. Twenty-five strains identified phenotypically were also identified by PCR/RFLP technique with outstanding difference between the two methods. Over expression of ERG-11 gene was observed in resistant and sensitive dose dependent (SDD) isolates as compared to the sensitive ones. PCR/RFLP technique is an accurate and rapid identification method as phenotypic methods had led to misidentification of *Candida* species. Over expression of ERG-11 gene in resistant and sensitive dose dependent (SDD) isolates highlights the importance of this gene in developing resistance to fluconazole and draws the attention to the necessity of extensive molecular studies on other mechanisms of resistance.

Keywords: *RFLP; Candida; Restriction Enzymes; ERG-11; E-Test; Azoles; Genotypic; Phenotypic*

Introduction

Candida species are a major contributor to morbidity and mortality in both pediatric and neonatal intensive care units Infections with *Candida* spp and coagulase negative *Staphylococci* are especially prevalent in both settings [1]. Infection with *Candida* spp is a leading infectious cause of death in children with cancer or following an organ or hematopoietic stem cell transplant [2,3]. *Candida* infections are frequent and major causes of septicemia in neonatal ICUs, and they are associated with high morbidity and mortality rates. Low birthweight preterm infants are especially vulnerable to these infections. The most frequently encountered fungal infections are caused by *Candida albicans* or *Candida parapsilosis* [4].

Neonatal *Candida* infection can be acquired by neonates either vertically through the vagina of their mothers during their birth or horizontally from the hospital environment through the hands of health care workers [5]. Consequently, vulvovaginal candidiasis is a major risk factor for neonatal colonization with *Candida*. Colonization of the new born could be the first step in the development of infection which could either be mucocutaneous or systemic [6,7]. The progression towards *Candida* infection depends on a number of factors including prematurity, low birth weight, total parenteral nutrition (TPN), prolonged intake of broad spectrum antibiotics and invasive procedures as central venous catheters [8].

Candida albicans has been the most frequently isolated species in both ICUs, however non-albicans *Candida* (NAC) have emerged as an important cause of *Candida* infection, particularly *Candida* tropicalis, *C. parapsilosis, C. krusei* and *C. glabrata* [9].

There is a global increasing usage of azoles antifungal agents due to their therapeutic activity against different *Candida* spp. Among the azoles drugs, fluconazole shows that satisfactory tolerance has appeared and antifungal drug resistance is quickly becoming a major problem especially in immuno-compromised patients [10].

As antifungal susceptibility often varies among different *Candida* species; several of NAC exhibit intrinsic resistance to triazoles like fluconazole. Thus, accurate identification of isolates at the species level and antifungal susceptibility is crucial for proper diagnosis and early treatment of invasive infections [11].

Conventional methods for identification of *Candida* species are based mainly on phenotypic features as carbohydrate assimilation, chromogenic media, germ tube test and morphology [12]. The set back of these methods is that they are time consuming; besides they may lead to mis-identification, particularly in the case of the closely related species [13].

Genotypic identification of *Candida* species represents a reliable alternative method and may improve species characterization, particularly in cases of the closely related species in the *Candida* complexes [13,14]. Identification of *Candida* species has been achieved by restriction fragment length polymorphism (RFLP) analysis of the ribosomal DNA (rDNA) repeat of *Candida* species [14-16]. Several molecular approaches have been developed and were designed mostly for the ribosomal RNA (rRNA) genes: targeting either the D1D2 domain of the 26S rRNA large subunit or the internal transcribed spacer regions ITS1 and ITS2 [12,14,15]. In addition to identification of *Candida* at species level; molecular methods also provide information about resistance to antimicrobials as fluconazoles [16].

Azoles inhibit fungal growth by interfering with the synthesis of ergosterol, a necessary component of fungal cell membranes. The ergosterol biosynthetic pathway is interrupted by azoles through inhibition of the enzymatic activity of 14- α -sterol demethylase (also known as CYP51A1), the product of the ERG11 gene. Azoles have a basic nitrogen that coordinates to the iron atom of the heme group located in the active site of 14- α -sterol demethylase. The active site is thus occupied by the azole, which acts as a non-competitive inhibitor [17]. It is important to note that the unrationalized use of Itraconazole and fluconazole is suggested to be the main reason of azole resistance. The innate resistance pattern for fluconazole is clear in *Candida kruesi*, *C. glabrata* and sometimes *C. tropicalis*. Some studies revealed that about one-third of patients with advanced AIDS had fluconazole-resistant *C. albicans* in their oral cavities; Based on data from the ARTEMIS Global Antifungal Surveillance Program, 2014 [18].

Several molecular mechanisms are involved in azole resistance which include (1) decreased affinity of azoles for the target enzyme CYP51A1 caused by point mutations in the ERG11 gene, (2) increases in CYP51A1 copy number through upregulation of ERG11 expression, (3) metabolic modifications, and (4) decreased intracellular azole accumulation by upregulation of multidrug transporters or drug sequestration [19-22].

Aim of the Study

This study aimed to identify *Candida* species in neonatal and pediatric ICUs by using PCR and RFLP analysis as well as screening of clinical isolates of *Candida* species especially those with resistance and susceptibility dose dependent patterns of sensitivity for over expression of ERG- 11 genes using RT- PCR technique.

Materials and Methods

This Study was conducted during the period from October 2015 till April 2016. Specimens included in this study were collected from in-patients at neonatal intensive care unit (NICU) and their mothers and inpatients from pediatric intensive care unit (PICU) of Ain Shams University Hospitals (ASUHs).

Subjects: Samples, per site of infection, were taken from neonates, infants and children suffering from signs of infection with one or more factors suggesting fungal origin. Samples included ascetic fluid, peripheral blood, central line, blood and urine. Maternal demographic, perinatal, and delivery data as well as neonatal and infant clinical data were collected. Clinical data included age, weight, cause of admission, duration of ICU stay, presence of neutropenia, drug intake including fluconazole and steroids. High vaginal swabs were obtained from mothers of neonates with *Candida* infection to investigate to what extent *Candida* isolates in neonates are similar to isolates from their mother's vaginal tract.

Sample collection and microbiological processing

Samples were collected after 48 hours of admission to either NICU or PICU. They were examined within 2 hours of collection. Twenty-five clinical isolates of different *Candida* spp. were obtained from different clinical specimens; 14 of which were neonatal. In addition, high vaginal swabs from mothers of neonates were also obtained. All the clinical specimens were collected with proper clinical and laboratory procedures [23].

After microscopic examination of direct films stained with Gram staining, specimens were cultured (or sub-cultured in case of blood samples) on Sabouraud dextrose agar (SDA), SDA supplemented with chloramphenicol and SDA supplemented with chloramphenicol and cycloheximide and on Brilliant CRHOM agar (Oxoid, UK) (Figure 1). Plates were incubated at 37oC aerobically for 24 to 48 hours then examined for candidal growth [24]. Identification of *Candida* species was done by microscopic examination, germ tube test and carbohydrate assimilation test (Auxacolor™ 2 kit).

Carbohydrate assimilation and fermentation tests and enzymes detection

Carbohydrate assimilation and fermentation tests and enzymes detection were done using the Auxacolor™ 2 kit (Bio-rad, France). All tests were performed according to the manufacturer's instructions. Inoculation of the wells was performed by adding a yeast suspension to the dehydrated substrates. The results were read after incubation for 24 and 48 h at 30°C. A five-digit numerical profile was generated for each isolate depending upon the reactions it produced. Two further digits were then added to the profile number depending upon the growth characteristics of the isolate in morphological tests. Identifications were made by referring to the list of numerical profiles provided by the manufacturer. Morphological characteristics essential for the Auxacolor profiles included pigmentation, arthrospores and capsule (Figure 2).

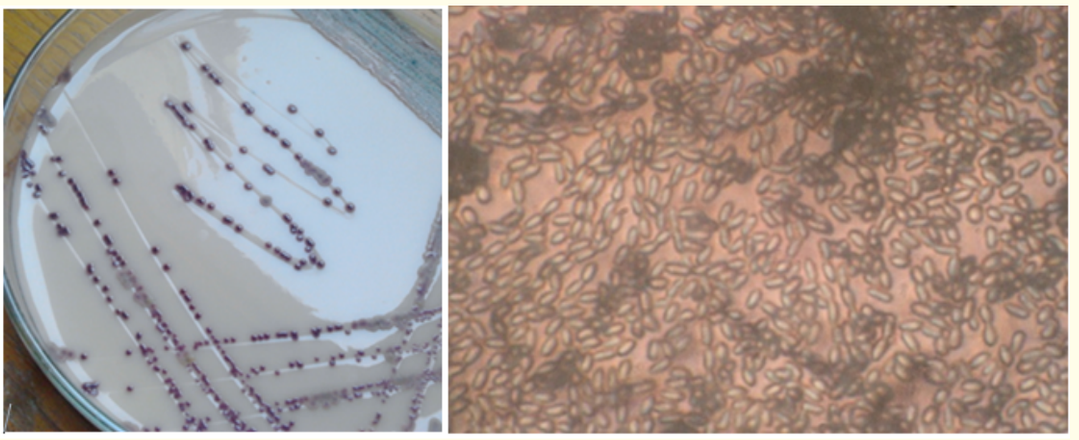

Figure 1: *C. krusei on Brilliant CRHOM agar (Oxoid: Production agency) yielding pink colonies (Left), On Corn meal examined microscopically showing elongated ovoid yeast cells (Right).*

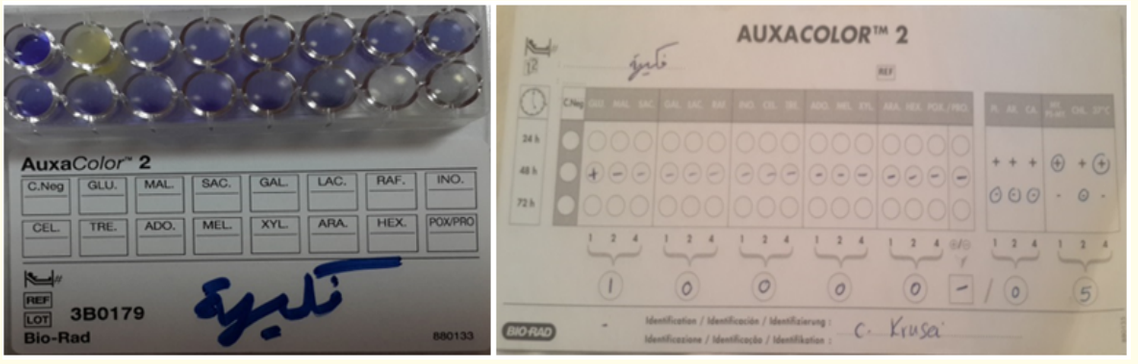

Figure 2: *C. krusei biochemical reactions on AUXACOLOR showing limited fermentative capacity for glucose only (Left) and AUXACOLOR coding sheet (Right).*

Determination of MIC of Fluconazole by E-test

Determination of the minimal inhibitory concentration of fluconazole for all Candida isolates was performed using the fluconazole E-test (Liofilchem, Italy) according to the CLSI guidelines [25]. The E test was performed by inoculation of a 150-mm petri plate containing 60 ml of agar with a sterile swab by using a cell suspension adjusted to a 0.5 McFarland standard. The E test strips were applied. The plates were incubated at 35°C and were read at 24 and 48h. The MIC was considered as the drug concentration at which the border of the elliptical inhibition zone intercepted the scale on the strip. MIC values were categorized as susceptible, susceptible dose dependent and resistant, as per table 1.

Molecular typing

DNA extraction: *Candida* isolates were grown on Sabouraud's Dextrose Agar for 48h at 30°C and suspended in sterile 0.9% NaCl solution until a 0.5 McFarland was obtained. Yeast suspensions were incubated with lysis buffer containing 0.1 mg/ml proteinase K, 150 mM NaCl, 25 mM EDTA, 10 mM Tris- HCl (pH 8.0), 0.5% SDS for 3h at 55°C. After the phenol chloroform extraction, pellet was kept overnight at -20°C in 95% ethanol with sodium acetate. The supernatant was discarded, and the pellet was allowed to dry. DNA was then dissolved in 50 µl of sterile water and 5 µl of DNA solution was used for amplification procedure [26,27].

Antifungal agent	Susceptible (S)	Susceptible-dose dependent (SDD)	Resistant (R)
Fluconazole	8 µg/ml	16 - 32 µg/ml	64 µg/ml

Table 1: *Interpretive breakpoints for Candida spp. and fluconazole.*

Amplification: PCR was used to amplify intergenic spacer regions (ITS) of ribosomal DNA (rDNA) with the primers ITS1 (5'-TCC GTA GGT GAA CGT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3'). PCR was performed in 50 µl reaction mixture using Qiagen Hatstat mastermix (a company for production of Master mix) as per described in kit handbook [28] containing 0.2 mM each deoxynucleoside triphosphate, 1.5 mM magnesium chloride, 0.5 µM each primer, 10x Taq buffer and 2.5 U of Taq polymerase (DNAmp, England) and 0.5 µg of candidal DNA as template were used. Negative controls were performed with sterile deionised water in place of the template DNA. PCR conditions for the amplification step were: denaturation at 95°C for 1 minute, primer annealing at 55°C for 1 minute and extension at 72°C for 15 minutes [14]. A Hybaid thermal cycler was used for the PCR reactions (Hybaid Corp., Cambridge, UK). Ten µl from the amplicons were analyzed in 2% agarose gel with 1x TBE buffer stained with ethidium bromide and visualized by illumination with UV light.

Typing by RFLP

PCR products were digested individually with 10 U of restriction enzymes HaeIII, BfaI, and DdeI (New England Biolabs, Hitchin, UK) by overnight incubation at 37°C. The resulting restriction fragments were analysed by agarose gel electrophoresis using 3% 3:1 NuSieve agarose (FMC BioProducts, Kent, UK) gels in 1x Tris-phosphate-EDTA buffer [14,29]. Analysis and identification of Candida isolates was performed. Four species were identified by the RFLP technique; *C. albicans, C. parapsilosis, C. krusei, C. glabrata.* The intergenic spacer region was amplified from all tested isolates, and a distinct product size was obtained for all isolates of a given species. All isolates yielding a product size of approximately 800 bp identified as *C. glabrata*. A product of approximately 520 bp was obtained from the remaining isolates. These isolates were identified by RFLP analysis following digestion of the PCR products by the restriction enzymes HaeIII, BfaI, and DdeI [27]. The size of the fragments obtained from the products of restriction enzymes are shown in table 2.

	Bfa I	***HaeIII***	***deI***
Candida albicans	-	90, 430	100, 420
Candida parapsilosis	-	40, 110, 390	-
Candida krusei	120, 200	40,90, 380	-
Candida glabrata	-	200, 650	-

Table 2: *Size of the fragments obtained from the products of restriction enzymes Bfa I HaeIII DdeI.*

Detection of ERG11-2 gene by Real Time-PCR

The real time PCR was done using SYBR® Green master mix (Thermo Scientific, U.S.A) and primer sets designed to amplify ERG-11 gene and (ACTIN) ACT1 (Biosearch technologies, U.S.A).

Real time PCR was done using one step machine (Applied Biosystems, USA) using Qiagen SYBR® Green master mix in 25 µl (0.5 µl from each primer (1% conc. For each), 10 µl from sample and the remaining amount consisted of master mix and water). The same was done using 0.5 µl forward and 0.5 µl reverse of ACT1 instead of those of ERG-11 primer.

The program used was for relative quantitative PCR by including the test group (isolates showing resistance and Sensitive dose dependent SDD to fluconazole) and the control group (isolates showing sensitivity to fluconazole) using gene specific primer and a house keeping gene (ACT1). The primers used span the entire ERG11 open reading frame: 5'-GTT GAA ACT GTC ATT GAT GG (forward) and 5'-TCA GAA CAC TGA ATC GAA AG (reverse) [30,31]. The primers for ACT1 were 5´-GAT TTT GTC TGA ACG TGG TAA CAG-3´ (forward) and 5´-GGA GTT GAA AGT GGT TTG GTC AAT AC-3´(reverse).

Cycling parameters included initial heating at 95°C for 15 minutes followed by 40 cycles of denaturation at 93°C for 30 seconds, annealing and extension at 60°C for 1 minute. Post samples were identified by Threshold cycler number (CT) value above the threshold level which is generated automatically by the machine. Specificities if the products were identified by melt curve analysis (Figure 3).

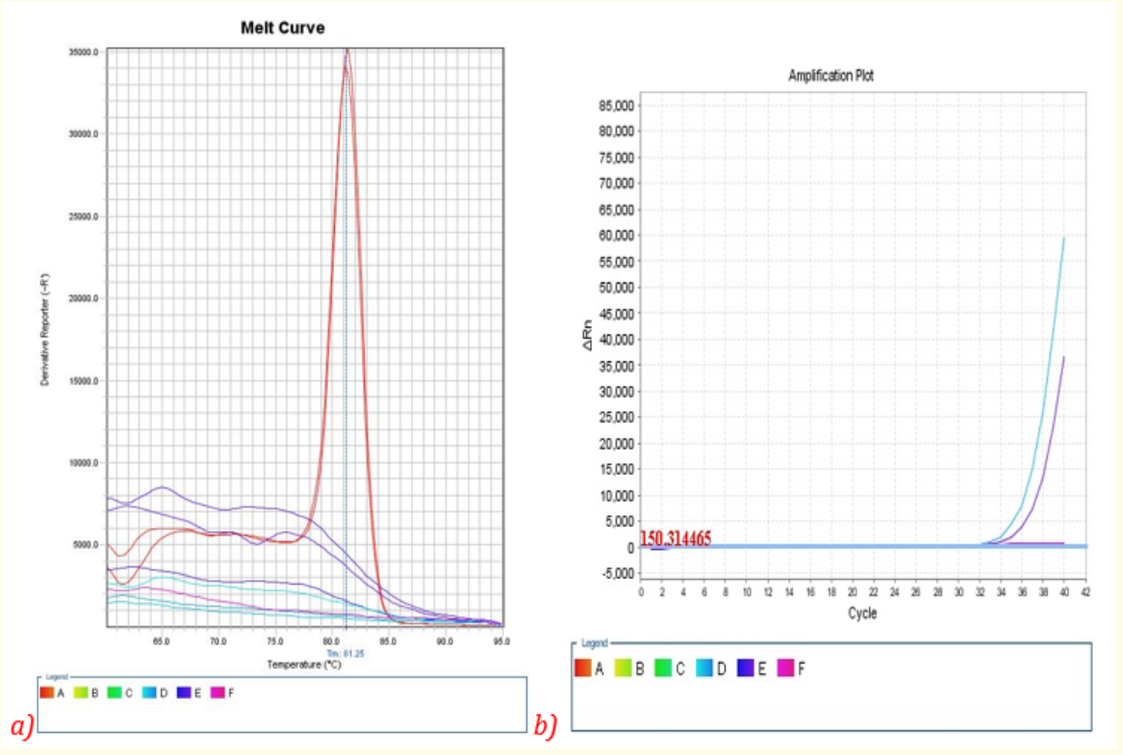

Figure 3: *These figures show the melt curve for drug resistance with the peak at 820C. The other low flat peaks are negative for the drug resistance gene.*

Data was collected, tabled and statistically analyzed using SPSS vs. 15. Parametric data was expressed as minimum, maximum, mean ± SD. Non-parametric data was expressed as number and percentage. Comparisons between two groups as regards non-parametric data were done using either Chi-square or Fisher exact test. Comparisons between more than two groups as regards non-parametric data were done using Chi-square test. Two tailed p value > 0.05 was considered insignificant, meanwhile p ≤ 0.05 was considered significant.

Ethics statement

Informed consents were obtained from informed mothers, parents or guardians. This work has been conducted after approval of Ain Shams University Ethical Committee and in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments in humans.

Results

Clinical characteristics of the studied subjects

Of the total 25 patients included in the study 15 (60%) were males and 10 (40%) were females. Their age ranged from 9 days to 3 years; 14 (56%) were less than 1 month, 4 (16%) were less than 1 year while 7 (28%) were more than one year. Clinical characteristics of the studied cases are presented in table 3.

Weight (gm)	
Min. – max.	1700 - 22000
Mean ± SD	6060 ± 5591.7
ICU type N (%)	
NICU	14 (56)
PICU	11 (44)
Cause of admission N (%)	
Dehydration	2 (8)
Diabetic coma	1 (4)
Malignancy	3 (12)
Hepatic coma	1 (4)
Congenital adrenal hyperplasia	1 (4)
Heart failure	1 (4)
Nephrotic S	2 (8)
Pneumonia	1 (4)
Post urethral valve	2 (8)
Prematurity	11 (44)
Patients with malignancies	2
Sample N (%)	
Ascetic fluid	1 (4)
Peripheral Blood	11 (44)
Central line blood	3 (12)
Urine	10 (40)
Duration of ICU stay (days)	
Min.-max.	7 - 22
Mean ± SD	13.84 ± 4.19
Neutropenia N (%)	5 (20)

Table 3: *Clinical characteristics of the studied cases.*

Isolated *Candida* species

Of the 25 isolated *Candida* species; 17 (68%), 3(12%), 1 (4%), and 4 (16%) were *C. albicans, C. glabrata, C. krusei* and *C. parapsilosis* respectively using RFLP method (Figure 4). The phenotypic method identified 9 (36%), 1 (4%), 4 (16%), 4 (16%) and 7 (28%) as *C. albicans, C. krusei, C. parapsilosis, C. kefyr* and *C. tropicalis* respectively (Table 4). The concordance between the 2 methods was 20% while the discrepancy was 80%.

Maternal factors of studied neonates

Of the total 14 neonates included in this study; 8 (57.1%) and 6 (42.9%) were delivered vaginally and by caesarian section (CS) respectively; 10 (71.4%) were positive for vaginal Candidal infection, while 4 (28.4%) were negative. Maternal factors are presented in table 5. The Candida species was identical in both the mother and her neonate in 8/10 (80%) of cases; 7 cases were vaginally delivered and only one case was delivered by CS ($p < 0.05$) (Table 6).

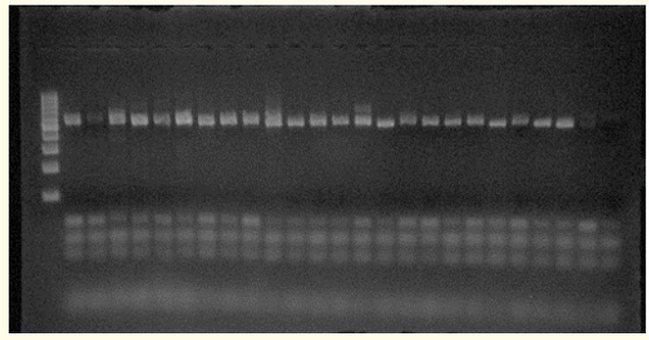

Figure 4a: *This figure shows the amplified product for the pan candida PCR using primers U1 and U2 with amplified product size at about 550 - 600 base pairs.*

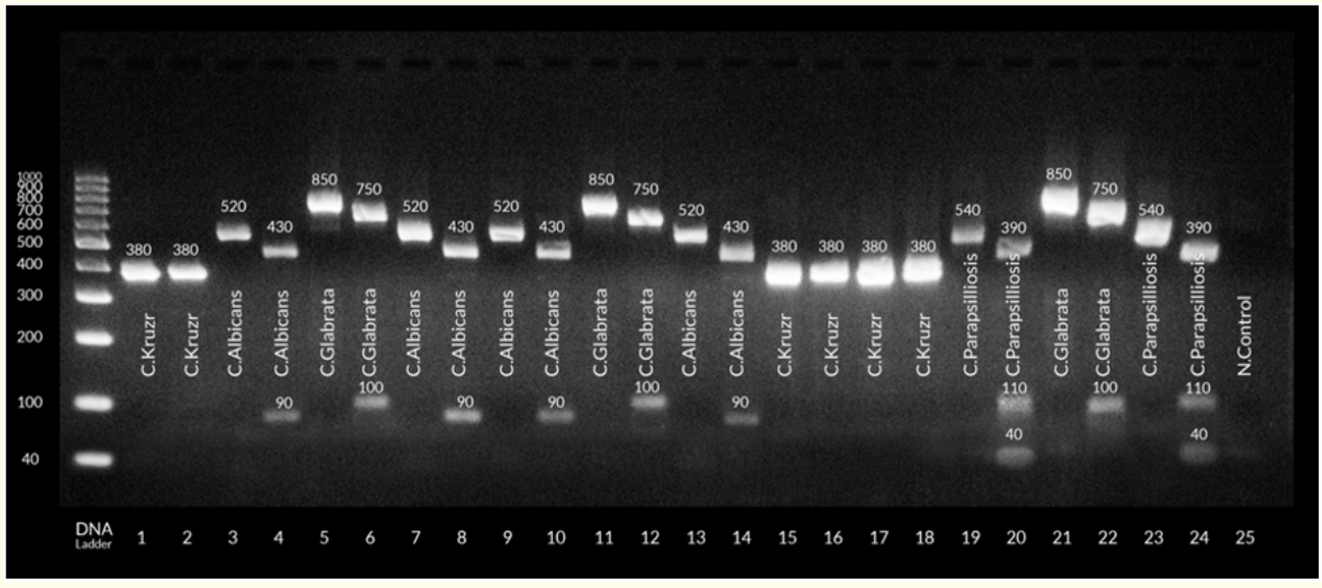

Figure 4b: *This figure shows the results of restriction enzyme cut after PCR each two adjacent bands represent one sample first lane before cut and the second lane after restriction digestion.*

Lane one represent DNA ladder

Land 2 and 3 represent Candida Cruzi which is not cut by the enzyme.

Lane 4 and 5 represent Candida Albicans un cut size 520 and the cut product at 430/90.

Lane 6 and 7 represent Candida C. glabrata un cut size 850 and the cut product at 750/100.

Lanes 8-11 represent Candida Albicans un cut size 520 and the cut product at 430/90.

Lanes 12 and 13, 22 and 23 represent Candida C. glabrata un cut size 850 and the cut product at 750/100.

	PCR/RPLP identification method	Phenotypic identification method	X^2	p
Albicans	17 (68)	9 (36)	5.12	0.02*
Glabrata	3 (12)	0	3.1	0.07
krusei	1 (4)	1 (4)	0	1
Parapsilosis	4 (16)	4 (16)	0	1
Kefyr	0	4 (16)	4.3	0.03*
Tropicalis	0	7 (28)	8.1	0.004*

Table 4: *Comparison between Candida species type by genotypic and phenotypic methods.*

p ≤ 0.05 significant

p > 0.05 insignificant

Mode of delivery	
Vaginal	8 (57.1)
Caeserian section (CS)	6 (42.9)
Maternal Candida infection	
Positive	10 (71.4)
Negative	4 (28.6)
Gestational diabetes mellitus	
Positive	6 (42.9)
Negative	8 (57.1)
Maternal steroid therapy	
Yes	2 (14.3)
No	12 (85.7)

Table 5: *Maternal descriptive data of NICU patients.*

Identical neonatal and maternal confirmed Candidal infection	Vaginal delivery	CS	p
Positive	7	1	0.02*
Negative	1	5	

Table 6: *Relation between maternal Candida infection and mode of delivery.*

p ≤ 0.05 significant

p > 0.05 insignificant

Fluconazole susceptibility of isolated *Candida* species

Of the 25 isolated *Candida* species; 6 (24%) were resistant by E-test, while 2 (8%) were sensitive dose dependent (SDD), 15 (60%) of cases were exposed to fluconazole (Table 7).

Sensitivity N (%)	
SDD	2 (8)
Resistant (R)	6 (24)
Sensitive (S)	17 (68)
Fluconazole exposure N (%)	
Yes	15 (60)
MIC	
≥ 256	4 (16)
0.5 - 256	7 (28)
≤ 0.05	14 (56)

Table 7: *Fluconazole susceptibility by E-test.*

The species distribution and *in vitro* susceptibility of *Candida* isolates is clarified in table 8. The prevalence of fluconazole resistance among *Candida albicans, glabrata, krusei,* and *parapsilosis*, was 23.53%, 33.33%, 100% and 0% respectively. One *C. albicans* isolate (5.88%) and one *C. parapsilosis* isolate (25%) were sensitive dose dependent (SDD). There was a statistically significant relation between exposure of the case to fluconazole and development of resistance by the phenotypic method (Table 9).

	S	**R**	**SDD**	**X^2**	**p**
Albicans n = 17	12 (70.59%)	4 (23.53%)	1 (5.88%)	0.3	0.8
Glabrata n = 3	2 (66.67%)	1 (33.33%)	0 (0%)	0.3	0.8
Krusei n = 1	0 (0%)	1 (100%)	0 (0%)		
Parapsilosis n = 4	3 (75%)	0 (0%)	1 (25%)		
X^2	6.06				
p	0.4				

Table 8: *E-test susceptibility pattern of Candida species identified genotypically to fluconazole.*

Exposure to fluconazole	**E-test**				
	S	**R**	**SDD**	**X^2**	**P**
Yes (n = 15)	7	6	2	7.8	0.02*
No	10	0	0		

Table 9: *Relation between E- test susceptibility results and exposure to fluconazole.*

The gene expression level of ERG-11 gene in resistant and sensitive dose dependent (SDD) isolates was higher than that of the sensitive ones, and the difference was statistically significant (Table 10).

	Resistant and SDD isolates	**Sensitive isolates**	**P**
	Mean ± SD	**Mean ± SD**	
Erg-11	98.3 ± 39.7	25.1 ± 12.8	0.04*

Table 10: *ERG-11 gene expression in sensitive isolates and in resistant and SDD isolates by RT-PCR.*

** Statistically significant at P-value ≤ 0.05.*

Discussion

Invasive *Candida* infections have increased significantly and represent a major concern in PICUs and NICUs. Although *C. albicans* remains the most frequently isolated *Candida* species, there has been a significant rise in isolation rates for non-albicans *Candida* species (NAC) [32,33]. This study showed that the most prevalent isolated species was *C. albicans* by both phenotypic and genotypic methods; 9 (36%) and 17 (68%) respectively. *C. tropicalis*, *parapsilosis* and *kefyr* come next by phenotypic method (28%, 16% and 16% respectively).

Our results come in accordance with Kuzucu., *et al.* 2008 in Turkey who found *C. albicans* to be the most prevalent species in both NICU and PICU followed by *C. parapsilosis* using phenotypic methods [34].

Similarly, in 2016, Novak and Pleško in Croatia conducted a retrospective epidemiologic mul¬ticentre pilot study in five neonatal and five pediatric intensive care units. The most common isolated *Candida* from those patients was *C. albicans*, the second most common was *C. parapsilosis* [35]. Roilides., et al. 2004 in Greece conducted a prospective observational study of invasive candidiasis in NICU; *C. albicans* was the most common species observed in 65.5% of cases, followed by *C. parapsilosis* in 15.5% and *Candida* tropicalis in 7% [32].

In contrast Juyal., et al. 2013 in India performed a study in NICU and found that NAC were responsible for 80.3% of *Candida* infection whereas *C. albicans* was isolated in only 19.7% of cases. *C. parapsilosis* (25%), *C. tropicalis* (21.97%) were the pre-dominant isolated *Candida* species [37].

Conventional methods for diagnosis of *Candida* infection often leads to misidentification especially among closely related species, phenotypic switching between different *Candida* species is also observed. This necessitated the development of molecular methods for accurate identification of *Candida* species. Restriction fragment length polymorphism analysis of the ribosomal DNA has been used in several studies for identification of a number of species at the same time which is an advantage over other molecular techniques which usually concentrates on a single species [14,27].

In the present study, PCR/RFLP method showed that *C. albicans* was the most prevalent isolated species (68%) followed by *C. parapsilosis* and *C. glabrata* with prevalence of 16% and12% respectively. Other studies that used RFLP identification method also recognized C. albicans as the most prevalent species followed by *C. glabrata* and *parapsilosis* [14,27,38]. In the same vessel, Shokohi., *et al.* 2009 in Iran used different endonuclease restriction enzymes; however, the study yielded similar results as regards prevalence of *C. albicans* among other *Candida* species [39].

Some species may show very similar phenotypic characters meanwhile genotypically they are significantly different. The genotype of a given species is a fingerprint which is impossible to be identical between two different strains [40]. Our results support this principle as 8 *C. albicans* isolates identified by RFLP/PCR were misidentified by phenotypic methods as *C. tropicalis* and *C. kefyr*; whereas, *C. glabrata* was completely missed by phenotypic methods.

In the same context, Comet., *et al.* 2011 clarified the same concept although their results were not similar to the present study. They concluded a genotypic difference between *C. albicans* and *C. dubliniensis* while both species showed same phenotypic characters. They observed that phenotypic methods have led to dramatic misidentification that occurred in very closely related species as *C. Guilliermondii* and *C. famata*. Moreover, our PCR/RFLP protocol had typically succeeded to identify *C. krusei* which was considered as a limitation in their study [13].

Regarding *C. parapsilosis*, it is mandatory to be identified as it has a different antifungal susceptibility. Misidentification of *C. parapsilosis* leads to treatment failure due to its resistance patterns to echinocandins. It gives better results with azole group (fluconazole). In our study, *C. parapsilosis* gave similar results by both phenotypic and PCR/RFLP methods [41].

Maternal vaginal candidiasis is an important risk factor for colonization of the newborn with *Candida* species which could be the first step in the development of *Candida* infection [42]. Therefore, vaginal delivery is also considered as an important risk factor for neonatal colonization with *Candida* species which could later develop into serious infection and is the most important predictive for neonatal invasive candidiasis [43,44].

Out of the 14 neonates included in our study; 11 were preterm, 8 were delivered vaginally while 6 were delivered by CS. *Candida* species isolated from neonates delivered vaginally were identical to *Candida* species isolated from the vagina of their mothers by both phenotypic and PCR/RFLP identification methods. While only one case delivered by CS fulfilled the same conditions and this difference between the two delivery methods was statistically significant.

These findings are supported by previous studies [43,44]; where vaginal delivery was identified as a risk factor for neonatal colonization with *Candida*. Benjamin., *et al*. 2010 performed a prospective observational cohort study at 19 centers in USA and recognized vaginal delivery as a possible risk factor for invasive candidiasis [45]. The preterm's colonization species were all identical to their mother vaginal colonization species. Similarly, Zisova., *et al*. 2015 found out that isolates of the newborns were 100% identical to those of the mothers' vaginal secretion by API *Candida* test [42]. Filippidi *et al*., 2013 studied Candida isolates from neonates and the vagina of their mothers. Candida genotyping by pulse field gel electrophoresis revealed identical strains in all investigated neonate–mother pairs [46].

Assessment of susceptibility have been implicated to determine whether an isolate is sensitive or resistant to the panel of commercially antifungal drugs. These methods include disc diffusion, Eucast, Fungitest, E test and microtitre dilution plates for MIC determination. The evolution of a widespread rapid antifungal sensitivity testing helps to save money, and supplies standardized, guided and excellent care. It is important to note that azole sensitivity testing is not an easy task and should be always verified and held by expertise. In 1997, the CLSI (previously NCCLS) provided guidelines for antifungal susceptibility testing in yeast (M27-A2/A3) and in filamentous fungi (M38-P) clarifying the threshold values of MIC above which an isolate is considered resistant [46].

As regards fluconazole susceptibility results by E-test; 6 isolates were resistant; 2 isolates were sensitive dose dependent (SDD) while the remaining 17 isolates were reported as sensitive. Out of the 17 *C. albicans* isolates; 23.53% were resistant while 5.88% were sensitive dose dependent (SDD). The one *krusei* isolate was resistant whereas 3 *parapsilosis* isolates were sensitive and one was sensitive dose dependent (SDD).

Many mechanisms of resistance for fluconazole exist in *Candida* species. Over expression of ERG-11 genes which encode for lanosterol 14α-demethylase is of particular interest in this study. Over-expression of ERG-11 gene may occur due to genetic point mutation in ERG-11 gene. The unique primers used in the present study gave a chance of easy identification as its design facilitates detection of ERG-11 gene point mutation [47].

In our study; the gene expression level of ERG-11 gene in resistant and sensitive dose dependent (SDD) isolates was significantly higher than that of the sensitive ones. Pam., *et al*. 2012 in Nigeria who investigated the role of ERG-11 gene in 28 *Candida* isolates from cases of vulvovaginitis. They detected ERG-11 gene in 2 resistant and 3 sensitive dose dependent (SDD) isolates [48]. In concordance with our results, Chen., *et al*. 2010 reported over expression of ERG-11 gene in some of the resistant Candida isolates [49]. Perea., *et al*. 2001 studied the over expression and point mutation of ERG-11 gene and demonstrated the up regulation of ERG11 genes in 35% of isolates. This up regulation was associated with point mutations in the ERG-11 gene. PCR amplification and sequencing of the ERG-11 genes showed 13 nucleotide changes that led to amino acid substitutions in the enzymes of the resistant isolates; point mutations were linked to increases in the MICs of fluconazole [29]. In contrast, White., *et al*. 2002 clarified that the level of ERG-11 gene expression did not differ between azole susceptible and resistant strains; the level of resistance did not appear to be related to resistance to azoles [50].

The discrepancy between results from different studies could be explained by the fact that diversity of mechanisms could result in a phenotype of azole resistance. Other genes could be involved in the resistance mechanism. Mutation or over expression of these genes could also lead to resistance to fluconazoles. These genes include CDR1, CDR2, MDR1 and FLU1. Other mechanisms of resistance also exist for the azole group [21]. The resistance mechanisms identified in susceptible and resistant isolates are not sufficient to explain resistance in clinical isolates [48].

Noteworthy, fluconazole resistance is strongly associated with continuous exposure to fluconazole which is considered as a selective pressure impact. Our findings support this hypothesis; as there was a statistically significant relation between exposure of the case to fluconazole and development of phenotypic resistance.

Conclusion

Studying the full patterns of susceptibility of *Candida* spp. affecting neonates in NICU or children in ICU is mandatory to evaluate the plan of treatment, thus, avoiding the treatment failure status. Some strains that show a phenotypic pattern of susceptibility may hide a genotypic pattern of resistance that can be induced under certain conditions as exposure to the drug azole. Prospectively, assessment of antifungal susceptibility especially to azole should be put in its proper slot in the identification map in order not to miss a resistance strain that might show an apparent false phenotypic pattern of susceptibility and vice versa. RFLP-PCR identification would be a high recommendation as an easy, feasible and reliable way of genotypic correct identification. Moreover, Rationalized use of antimicrobials generally and antifungal drugs specially should be highly encouraged. Such use must be guided by sensitivity testing *in vitro* in order not to lose one of our weapons against infection. Adherence to CLSI guide lines and local guidelines is important to control the problem of resistance stressing on avoiding cross resistance regimens.

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