Frequency of *Candida* Species in Iranian Pediatric Heart Transplant Recipients with and without Diarrhea using PCR-RFLP and Sequencing

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ABSTRACT

Background: *Candida* species are known as one of the most prevalent agents causing opportunistic gastrointestinal infections in immunodeficient patients.

Objective: In this study, species distribution of *Candida* was determined in pediatric heart transplant recipients with and without gastrointestinal symptoms.

Methods: In this study, 59 stool samples were collected from heart transplant recipients including patients with and without gastrointestinal symptoms aged between 1-17 years. The patients underwent heart transplant surgery at Rajaei Cardiovascular, Medical & Research Center, in Tehran. Sabouraud dextrose agar was used for the initial culture, and CHROMagar *Candida* media were used for initial differentiation. Definitive species identification of *Candida* isolates was performed using PCR-RFLP and sequencing following DNA extraction.

Results: In the present study 13 (22 %) overgrowth isolates associated with patients with gastrointestinal symptoms were positive for *Candida* spp. using microscopy and culture. The *Candida* spp. including *C. albicans* 5 (38.5%), *C. glabrata* 4 (30.7%), *C. guilliermondii* 2 (15.4%), and mixed infection of *C. albicans* and *C. glabrata* 2 (15.4%) were identified.

Conclusion: *C. albicans* was the predominant species in pediatric heart transplant recipients with *Candida* intestinal colonization that was substantiated by the presence of pseudohyphae on microscopy, which could be consider as an evidence of invasion.

KEYWORDS: *Candida*; PCR; Pediatric; Transplant; RFLP; Frequency

INTRODUCTION

Candida species are one of the most opportunistic fungal agents that cause gastrointestinal infections especially in immunodeficient patients. Intestinal candidiasis with endogenous or exogenous origin can be occurred following antibiotic therapy [1]. Antifungal prophylaxis may be effective for prevention of candidiasis; however, antimicrobial resistance issues should be considered when using preventive protocols and guidelines [2].

Host and environmental situations are two factors that determine invasive fungal infections

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in immunodeficient patients. These factors include environmental exposure or colonization with endogenous origin, and prophylaxis with antifungal or immunosuppressive treatment. Primary and acquired immunodeficiencies patients including common variable immunodeficiency (CVID), chronic granulomatous disease, Human immunodeficiency virus (HIV), solid organ transplant (SOT) recipients, leukopenia or patients with underlying diseases, cirrhosis, and diabetes mellitus are at risk for invasive fungal infections [3-5].

Invasive fungal infections are an important cause of morbidity and mortality in SOT recipients [6, 7]. The incidence of invasive fungal infections has been reported almost 3% among SOT recipients in the first year after transplantation; however, this rate depends on the type of organ transplant [6].

Candidiasis is the most prevalent invasive fungal infection in SOT recipients and it is responsible for 50–60% of fungal infections [6]. *Candida* spp., especially *Candida albicans*, are found in gastrointestinal, skin and reproductive tracts in human. The most of invasive candidiasis is usually derived from the skin or gut as an endogenous origin [5].

The incidence of fungal infection in patients receiving a heart transplant varies between 10% - 25%. [8]. So, intestinal *Candida* colonization in pediatric heart transplant recipients can be helpful to understand the relationship between development of invasive *candida* in immunosuppressive hosts that leads to the improvement of patients' quality of life.

This study aimed to evaluate the incidence rate of intestinal *candida* in pediatric heart transplant recipients with and without diarrhea and determination the species distribution using PCR-RFLP and sequencing.

MATERIALS AND METHODS

Patients Sampling

In this study, 59 stool samples of patients receiving heart transplantation aged between 1-17 years were collected. The patients underwent heart transplant surgery in Rajaei Cardiovascular, Medical & Research in Tehran during 2019-2020. The demographic information and clinical characteristics including age, gender, and heart disease type leading to transplantation, date of heart transplantation, clinical manifestation, gastrointestinal disorder and immunosuppressive drugs were collected. Heart transplantation recipients underwent on tacrolimus, prednisolone and cellcept. All transplant recipients were on oral administration of TMP-SMX from the first day of transplantation as a prophylaxis treatment for preventing *Pneumocystis jiroveci* and *Toxoplasma gondii*.

The stool samples of patients with gastrointestinal symptoms (GI) including diarrhea, nausea, and without GI symptoms were collected. The stool specimens were then transferred to the Research Center of Pediatric Infectious Diseases, Iran University of Medical Sciences.

All of the patients were evaluated for parasites and bacterial infection. The stool samples were examined by wet mount examination with Phosphate-buffered saline (PBS) and the formalin ether concentration method for diagnosis of trophozoites and cysts of parasites. Additionally Trichrome staining was utilized to provide more confirmation. Lastly, the modified acid-fast staining method was performed to detect coccidia like *Cryptosporidium* and *Isospora belli*.

The chromotrope staining method was performed for detection of microsporidia spores and finally the stained specimens were observed by a light microscope. Additionally, the stool samples were cultured and analyzed using media for primary isolation and enrichment for bacteria. Furthermore, Tacrolimus serum levels were checked for all pediatric heart transplant patients and Tacrolimus serum level was normal for all patients to remove the confounding factors.

The stool samples were cultured on sabouraud dextrose agar (SDA, QLAB) and incubated at 35°C for 48 hours and the yeast growth was

Table 1: Comparison of frequency between different age groups with and without gastrointestinal symptoms[a]

		Gastrointestinal signs		P-value
		Yes	No	
	Mean ± SD	8.53 ± 4.57	9.71 ± 3.99	0.36[b]
Age groups	<5 years	4 (30.8)	8 (17.4)	0.94[b]
	6-10 years	4 (30.8)	16 (34.8)	
	11-15 years	4 (30.8)	19 (41.3)	
	>15 years	1(7.6)	3 (6.5)	
	Total	13	46	

[a]Values are expressed as No. (%)
[b]Chi-square

first detected and confirmed by gram staining method and then the isolates were cultured on CHROMagar Candida (Merck, Germany) and incubated at 35°C for 48 h to detect and differentiate species according to the colony color appearance.

DNA Extraction

Genomic DNA was extracted using a loop full of fresh clinical isolates was suspended in 200 µl of lysis buffer according to manufacture protocol (Roche Diagnostics GmbH, Mannheim, Germany).

PCR Method

Internal transcribe spacer (ITS) of ribosomal DNA was target for amplification. PCR was carried out in 25 µl as final volume. Each reaction contained 3 µl of template DNA, 20 pmol each forward (ITS1, 5'-TCC GTA GGT GAA CCT GCG G-3') and reverse (ITS4, 5'-TCC TCC GCT TAT TGA TAT GC-3') primers, 12.5 µl mastermix PCR (yektatajhiz, Iran). PCR condition including an initial denaturation at 94°C for 5 min then was followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 45 s, extension at 72°C for 1 min, and a final extension at 72°C for 7 min [9, 10]. PCR products were electrophoresed on agarose gel using a 1 % agarose gel in TBE buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA, pH 8.3) and visualized under UV irradiation. In the following, the results of gram staining, SDA culture and PCR were compared in the patients with and without gastrointestinal symptoms in term of the detection of yeast agents.

RFLP Analysis

Digestion was performed by incubating a 10 µl of PCR product and 2 µl of *MspI* (10U) (Thermo Fisher Scientific, USA) at final reaction volume of 30 µl at 37°C for 2 h. Restriction fragments were electrophoresed on 1.5% gel agarose [11].

Sequencing

A second round of PCR was performed for *Candida* positive isolates and direct sequencing using the dye termination method and an ABI 3730xl sequence was done, all of the sequences were analyzed using Genius software (version 7.0) and obtained sequences were blasted. The result of sequencing was compared with PCR-RFLP and CHROMagar for different *Candida* spp.

Ethical Considerations

The study was conducted after receiving approval from the ethics review committee of Iran University of Medical Sciences (IR.IUMS.REC.1399.773). Informed consent was taken from their children's parents. All patients' parents who participated in this study were informed about the study procedures and agreed to participate in this study. All methods were carried out according to relevant guidelines and regulations.

Statistical Analysis

The analysis was done using SPSS version 18 (Chicago, IL, USA), and chi-square tests were used to analyze the statistical relationship.

Table 2: Frequency of heart problems in the patients with and without gastrointestinal signs[a]

Transplant reasons	Gastrointestinal signs	Without gastrointestinal signs	Total	P-value
DCM	7 (53.8)	36 (78.3)	43 (72.9)	0.4[b]
FDCM	4 (30.8)	6 (13.1)	10 (16.9)	
RCM	1 (7.7)	1 (2.1)	2 (4.3)	
Long QT + TFTC	1 (7.7)	1 (2.1)	2 (4.3)	
RCM + HCM	-	1 (2.1)	1 (2.2)	
NCLV + CHB	-	1 (2.1)	1 (2.2)	
Total	13	46	59 (100)	

[a]Values are expressed as No. (%)
[b]Chi-square

RESULTS

Patients' Characteristics

A total of 59 pediatric heart transplant patients ranging between 1-17 years with a mean age of 9.45±4.12 participated in this study including 34 (57.6%) and 25 (42.4 %) male and female cases, respectively.

The mean ages of patients with and without gastrointestinal symptoms were 8.53±4.57 and 9.71±3.99 years, respectively. There was no significant difference in the mean age of patients with and without gastrointestinal symptoms (p=0.36). No significant difference was also observed between patients with and without gastrointestinal symptoms between age groups (p=0.94) (Table 1).

In this study, 13 (22%) patients had gastrointestinal symptoms while 46 (78%) cases had no gastrointestinal symptoms. The most common gastrointestinal symptoms were diarrhea (100%), abdominal pain (46.1%), and vomiting (23.1%). All of the patients were evaluated for parasites and bacterial infection and the patients were negative for enteric pathogens according to our investigation.

The most common type of underlying heart disorders was dilated cardiomyopathy (DCM) 43(72.9%), and familial dilated cardiomyopathy (FDCM) 10(16.9%). DCM was the most common cardiac disorder in both groups with 7 (53.8%) and without gastrointestinal symptoms 36(78.3%). There was no significant difference between patients with and without gastrointestinal symptoms and heart disorders (p=0.4) (Table 2).

Detection and Identification of *Candida* spp. by Culture Methods

Wet mount evaluation of patients with diarrhea indicated overgrowth of yeasts and budding pattern of yeast organisms. Furthermore, the detection of *Candida* was according to colony macroscopic (including white or creamy, round, soft, and smooth to wrinkle colonies) and microscopic (including the presence of round cells and pseudomycelium) features on sabouraud dextrose agar. The result of chrome agar for *Candida* spp. was compatible with standard color specious. In the present study, 13 isolates of *Candida* spp. (22%) as overgrowth was isolated from heart transplant patients with diarrhea, using a microscope, gram staining and culture on SDA. The *Candida* isolated from the stools of patients without diarrhea was low (n= 2) with a scanty dispersed colony. The frequency of *Candida* in patients with diarrhea was higher than in patients without diarrhea and differences were statistically significant (p<0.0001).

Identification of *Candida* spp. by Molecular Methods

In this study, fungus-specific universal primer pairs (ITS1 and ITS4) were used and PCR products between (510 to 870 bp) were produced according to the implication of ITS1-5.8S-ITS2 region of different clinical isolates (Fig. 1).

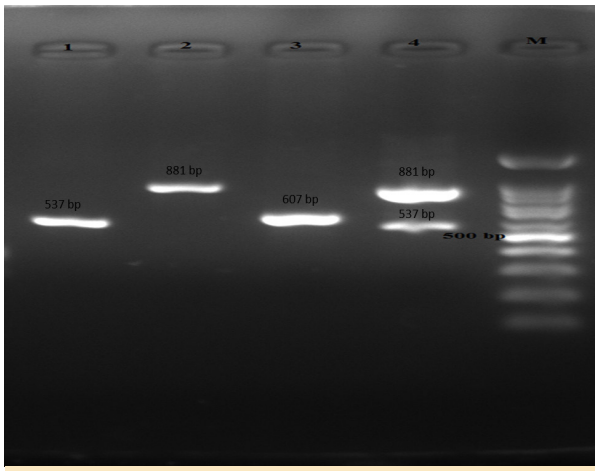

Figure 1: PCR products from *Candida* spp., 1: *C. albicans*, 2: *C. glabrata*, 3: *C. guilliermondii*, 4: Mixed infection of *C. albicans* and *C. glabrata*.

Fig. 2 demonstrates an example of the variation of *Candida* spp. identified by PCR-RFLP after digestion by the *MspI* enzyme. In general, digestion of the amplified product with *MspI* was according to the pattern of previous study[9].

In present study the results of PCR-RFLP for identification of the *Candida* spp. were compatible with the result obtained using CHROMagar Candida.

In general in patients with diarrhea 13 isolates of *Candida* spp. including *C. albicans* 5 (38.5%), *C. glabrata* 4(30.7%), *C. guilliermondii* 2(15.4%), and mixed infection of *C. albicans* and *C. glabrata* 2 (15.4%) were identified using PCR-FRLP and sequencing. The *C. glabrata* isolated from the stools of patients without diarrhea was 2(4.3%) with a scanty dispersed colony and the result was confirmed by PCR and sequencing (Table 3).

Demographic and clinical data of pediatric heart transplant recipients with *Candida* spp. infection was summarized in Table 4.

Sequence data of ITS region were deposited in the GenBank, and accession numbers of sequences are available for all isolates as follows: OK054543 - OK054547, OK091175- OK091180 and also OL819878- OL819879.

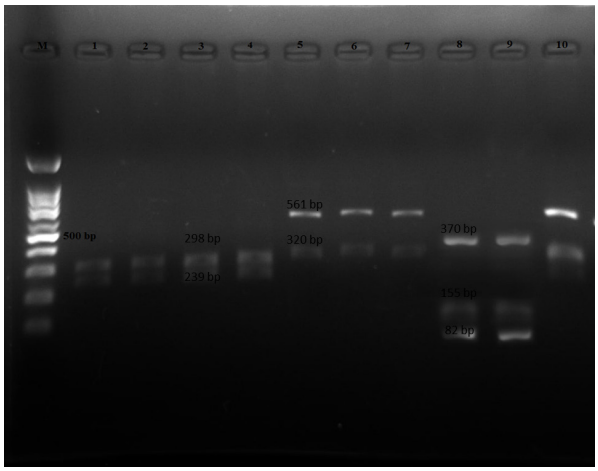

Figure 2: Restriction digestion of PCR products of *Candida* spp. with the enzyme *MspI*. Lanes 1-4: *C. albicans*, 5-7: *C. glabrata*, 8-9: *C. guilliermondii*, 10: Mixed infection of *C. albicans* and *C. glabrata*

DISCUSSION

Fungi, previously considered as commensals, are now emerging as opportunistic pathogens, especially in immunodeficient patients. The incidence of invasive fungal infections, including those of the gastrointestinal tract, has increased significantly as the number of immunocompromised patients has increased [12]. PCR-RFLP method using primers, ITS1 and ITS4, and digestion of the amplification product with *MspI* produced specific patterns for diagnosing the six different common *Candida* spp [11].

This study is the first report of the frequency of *Candida* spp. in Iranian pediatric heart transplant patients using molecular method; however parasitic infection assay was previously described [13].

In the present study 13 isolates of *Candida* spp. including *C. albicans* 5 (38.5%), *C. glabrata* 4 (30.7%), *C. guilliermondii* 2(15.4%), and mixed infection of *C. albicans* and *C. glabrata* 2(15.4%) were identified among pediatric recipients with diarrhea and *C. glabrata* isolated from the stools of patients without diarrhea was 2 (4.3%) with a scanty dispersed colony using PCR-FRLP and sequencing.

Invasive fungal infections have been reported

Table 3: Frequency of *Candida* spp. from pediatric heart transplant recipients with and without diarrhea[a]

	Patients with diarrhea	Patients without diarrea	P-value
***Candida* species**	**Number (%)**	**Number (%)**	
C. albicans	5(38.5)	-	**0.0001**[b,c]
C. glabrata	4(30.7)	2(4.3)	
C. guilliermondii	2(15.4)	-	
C. albicans and C. glabrata	2(15.4)	-	
Total	13(22)	46(4.3)	

[a]Values are expressed as No. (%)
[b]Chi-square
[c]statistically significant

in 25.5–59% of the intestinal transplantation recipients, with the most prevalent of *Candida* spp. [14-16]. The frequency of *Candida* spp., among intestinal transplantation recipients in the previous study and results of the two global surveillance monitoring systems, indicated *C. albicans* and *C. glabrata* being the dominant species in patients with fungemia, urinary tract infections and intra-abdominal infections [6, 14, 17].

Florescu *et al.* have reported 59 cases of candidiasis infection, including 39 (66.1%) fungemia and 17 (28.8%) with intra-abdominal abscesses or peritonitis in the intestinal transplantation recipients. The Frequency of *Candida* spp. among intestinal transplant recipients was as follows *C. albicans* 37.3%, *C. glabrata* 25.4%, *C. parapsilosis* 13.6%, *C. tropicalis* 5.1%, *C. krusei* 6.8%, and *C. lambica* 1.7% [14].

In a global surveillance study frequency of *Candida* spp. among organ transplant recipients was reported and results indicated *C. albicans* (46.2 %), *C. glabrata* (24.8%), *C. parapsilosis* (8.6%), *C. tropicalis* (4.4%), and *C. krusei* (2%). The most common invasive candidiasis was related with candidemia (64%), urinary tract (11%), and peritonitis (9%) [6].

The predominance of *C. albicans* between species obtained in this study is in line with some previous studies [18, 19]. The result of the present study is consistent with Naeini *et al.*, who reported intestinal *Candida* spp. infection (22%) as the most common fungal infection in kidney transplant recipients in a study [20].

Candida infections of the small and large intestines of patients with advanced cancer have been reported in a study [21]. Candidiasis associated with diarrhea has been reported in a renal transplant recipient. The esophageal, duodenal, and jejunal candidiasis was identified and ante mortem diagnosis of small bowel involvement was confirmed with radiologic and endoscopic results [22].

Candida infection has been reported from a 60-year-old man with kidney transplantation who has suffered from watery diarrhea for six months. Gastrointestinal endoscopy results confirmed esophagus and duodenum candidiasis. Biopsy results indicated active duodenitis with hyphal and yeast forms of *Candida* covering the duodenal epithelium in periodic acid Schiff (PAS) staining [23].

A total of 257 stool samples including 203 from diarrheal and 54 asymptomatic in Nigerian children were evaluated for *Candida* infection. Results indicated 165 (64.2%) cases had fungal infection. *C. albicans* had the highest prevalence (59.4%), then *C. tropicalis* (30.9%), *C. pseudotropicalis* (5%) in symptomatic children, and *C. glabrata* (3.0%), and *C. parapsilosis* (1.8%) in asymptomatic ones. Results indicated the emergence role of *Candida* spp. in children with diarrhea [18].

A total of 155 diarrheal stool samples were evaluated for *Candida* infection. *Candida* spp. was identified in 15 (9.7%) cases including, *C. albicans* (46.7%), *C. tropicalis* (33.3%), *C. krusei* (13.3 %), and *C. glabrata* (6.7%).

Table 4: Demographic and clinical data of pediatric heart transplant recipients with *Candida* spp. infection.

Patient no.	Age/ sex	Treatment	Rejection history	Underlying disease	Heart disorder	Gastrointestinal signs
1	15/M	tacrolimus, prednisolone, cellcept	-	-	FDCM	diarrhea
2	8/M	tacrolimus, prednisolone, cellcept	-	-	DCM	diarrhea
3	5/M	tacrolimus, prednisolone, azathioprine	-	-	FDCM	diarrhea, abdominal pain
4	13/M	tacrolimus, prednisolone, cellcept,	+ATG	GHD	DCM	diarrhea, abdominal pain
5	2/F	tacrolimus, prednisolone, cellcept	-	-	DCM	diarrhea
6	3/M	tacrolimus, prednisolone, cellcept, sirolimus	-	-	DCM	diarrhea, abdominal pain
7	11/M	tacrolimus, cellcept, sirolimus	-	LGMD	Long QT + TFTC	diarrhea, abdominal pain
8	10/M	tacrolimus, prednisolone, cellcept	-	-	DCM	diarrhea
9	10/F	tacrolimus, prednisolone, cellcept, sirolimus	+ ATG	-	DCM	diarrhea, vomiting
10	8/M	tacrolimus, prednisolone , cellcept	-	-	DCM	diarrhea, vomiting
11	14/M	tacrolimus, prednisolone , cellcept	-	HLH	FDCM	diarrhea
12	11/M	tacrolimus, prednisolone , cellcept	-	DM	RCM	diarrhea, vomiting abdominal pain
13	1/F	tacrolimus, prednisolone , cellcept	-	-	FDCM	diarrhea, abdominal pain

Abbreviations: antithymocyte Globulin (ATG); growth hormone deficiency (GHD); limb-girdle muscular dystrophy (LGMD); hemophagocytic lymphohistiocytosis (HLH); diabetes mellitus (DM); familial dilated cardiomyopathy (FDCM); dilated cardiomyopathy (DCM); tetralogy of fallot total correction (TFTC); restrictive cardiomyopathy (RCM)

Results indicated the presence of pseudomycelium in 14 (93.3%) culture-positive stools as a sign of invasion [19].

In another study, 314 pediatric cases were evaluated for *Candida* infection and the results demonstrated 110 (35.0%) cases of positive stool samples for *Candida* spp. *C. albicans* had the highest prevalence (59.1%), followed by *C. parapsilosis* (19.1%), and *C. tropicalis* (16.4%), while *C. krusei* had the least prevalence (5.5%), and 53 (48.2%) cases with mixed infection were found [24].

Candida was isolated as overgrowth from the stools of 32 (28.8%) non-HIV Patients and *Candida* isolated from the stools of healthy control was scarce. The frequency of isolates were *C. tropicalis* (n= 16), *C. albicans* (n= 14), and *C. krusei* (n= 2) and the result indicated *Candida* spp. may have an essential role in antibiotic-associated diarrhea [1].

Of 184 diarrheic stool cultures (35.9%) showed overgrowth of *C. albicans* and the result indicated association of *Candida* overgrowth to diarrhea was significant[25].

Overgrowth of *Candida* in antibiotic-associated diarrhea in comparison with healthy control was reported and *Candida* counts significantly had higher levels in antibiotic-associated diarrhea than in those from healthy control [26].

In the present study, overgrowth of *Candida*

and signs of invasion like budding in the yeast-like cells and the presence of pseudomycelium were found that to be compatible with the result of some studies that the presence of pseudomycelium as a marker for invasion of *Candida* in patients with diarrhea [1, 18, 27, 28].

The role of *Candida* causing diarrhea has been studied by some previous studies and *Candida* is strongly associated with diarrhea to be supposed as an enteropathogen [18, 29]; however, others believe that despite the cases of *Candida* found with diarrhea, further studies are needed to prove this point [19, 30].

In the present study, some transplant recipients with *Candida* infection had underlying diseases like diabetes mellitus, hemophagocytic lymphohistiocytosis, growth hormone deficiency, and Limb-girdle muscular dystrophy and some had rejection history and received antithymocyte globulin.

Although diarrhea in transplant patients is generally attributed to the immunosuppressive treatment, it can be due to other reasons like, infections [31-33], concomitant medicine [34], and underlying disease (diabetes mellitus, uremia) [35]. Results of a study indicated large percentage of severe diarrhea among renal transplant recipients is not related to immunosuppressive therapy and diarrhea was resolved after treatment for infections and changes to concomitant medicine [36].

Despite the cases of *Candida* found with diarrhea in the present study, further studies are needed to evaluate the association between *Candida* and diarrhea. The result indicated that PCR-RFLP used in the study is a proper method for diagnosing *Candida* spp. in the clinical isolates.

In conclusion, predominance of *C. albicans* between species was found in the present study and substantiated by the presence of pseudomycelium on microscopy, which is indirect evidence of invasion. Although the cases of *Candida* were found with diarrhea in the present study, further studies are needed to evaluate the association between *Candida* and diarrhea.

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