

Candidiasis in Pediatrics; Identification and *In vitro* Antifungal Susceptibility of the Clinical Isolates

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Abstract

Background

Candida species are normal microflora of oral cavity, vagina, and gastrointestinal tract. They are the third most prevalent cause of pediatric health care-associated bloodstream fungal infection. This study aimed to provide an epidemiological feature of candidiasis and also presents an antifungal susceptibility profile of clinical *Candida* isolates among children.

Materials and Methods

During July 2013 to February 2015, 105 patients from different hospitals of Isfahan, Iran, were examined for candidiasis by phenotypic tests. Samples were obtained from nail clippings, blood, thrush, BAL, urine, oropharynx, skin, and eye discharge. The age range of patients was between 18 days to 16 years. Genomic DNA of isolates was extracted and ITS1-5.8SrDNA-ITS2 region was amplified by ITS1 and ITS2 primers. The PCR products were digested using the restriction enzyme *MspI*. Minimum inhibitory concentration (MICs) was determined using microdilution broth method according to

the clinical and laboratory standards institute (CLSI) M27-A3 and M27-S4 documents.

Results

Forty-three patients (40.9%) had *Candida* infection. The most clinical strains were isolated from nail infections (39.5%), and candidemia (13.9%). *Candida albicans* was the most prevalent species (46.5%). MICs ranges for amphotericin B, fluconazole, and itraconazole were (0.025-0.75 µg/ml), (0.125-16 µg/ml), and (0.094-2 µg/ml), respectively.

Conclusion

Due to high incidence of *Candida* infections among children, increasing of fatal infection like candidemia, and emergence of antifungal resistance *Candida* isolates, early and precise identification of the *Candida* species and determination of antifungal susceptibility patterns of clinical isolates may lead to better management of the infection.

Keywords

Antifungal susceptibility, Candidemia, Pediatrics

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Introduction

The prevalence of fungal infections has increased since the 1980s in different patient groups, particularly in young, immunosuppressed, and hospitalized patients, and connected to extra morbidity and mortality. *Candida* species are normal

microflora of oral cavity, vagina, and gastrointestinal tract (1-3), and are responsible for different clinical forms of the infection, from mucocutaneous colonization to bloodstream fatal infections, for example *Candida* species are the third most prevalent cause of

pediatric health care-associated bloodstream fungal infection in the United States and Europe (4). Candidosis results from an endogenous colonization; however, nosocomial transmission and resistant strains to antifungal agents propose new and remarkable problems (5). This investigation sets an epidemiologic study focusing on the etiologic agents of candidiasis in pediatrics and antifungal susceptibility pattern of *Candida* species due to various clinical forms of candidiasis among this population.

Materials and Methods

Isolates

A total of 105 patients with suspected candidiasis were included in this cross-sectional study, from different hospitals of Isfahan, Iran, during July 2013 to February 2015. Specimens were collected from nail clippings, blood, thrush, BAL, urine, oropharynx, skin, eye discharge, and sore. All specimens were examined by direct microscopic examination with 15% potassium hydroxide (KOH), and culture on sabouraud glucose agar (Difco, Detroit, MI, USA), and CHROMagar *Candida* (Paris, France).

Molecular identification

DNA extraction

The genomic DNA of all isolates was extracted according to the previously described phenol-chloroform method using boiling technique (6). Briefly, a piece of fresh and single colony was added to the 1.5 ml Eppendorf tube containing 300 μ l of lysis buffer (200 mM Tris-HCl (pH 7.5), 25 mM EDTA, 0.5% w/v SDS, 250 mM NaCl). The suspension was mixed with phenol chloroform, and centrifuged at 10,000 g for 10 min. DNA was precipitated with an equal volume of isopropanol and 0.1 volume of 3.0 M sodium acetate (pH 5.2) and the DNA pellet was washed with 70% ethanol, dried in air, suspended in 50 μ l of distilled water and kept at -20°C.

PCR-RFLP

The ITS1-5.8SrDNA-ITS2 region was amplified using PCR mixture including 5 μ l of 10 \times reaction buffer, 0.4 mM

dNTPs, 1.5 mM MgCl₂, 2.5 U of Taq polymerase, 30 pmol of both ITS1 (5' - TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5' - TCC TCC GCT TAT TGA TAT GC-3') primers (7) and 2 μ l of extracted DNA in a final volume of 50 μ l. During the second step, PCR products were digested with the restriction enzyme MspI (Fermentas, Vilnius, Lithuania). Five and 12 μ l of each PCR and RFLP products were separated by gel electrophoresis on 1.5 and 2% agarose gel (containing 0.5 μ g/ml ethidium bromide), respectively.

In vitro antifungal susceptibility testing Minimum inhibitory concentration (MICs) was determined according to the recommendations stated in the clinical and laboratory standards institute (CLSI) M27-A3 and M27-S4 documents. Amphotericin B (AmB; Bristol-Myers-Squib, Woerden, The Netherlands), fluconazole (FLU; Pfizer Central Research, Sandwich, United Kingdom), and itraconazole (ITC; Janssen Research Foundation, Beerse, Belgium) were used for preparation of the CLSI microdilution trays. The antifungal agents were diluted in the standard RPMI-1640 medium (Sigma Chemical Co.) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) (Sigma) with L-glutamine without bicarbonate to yield two times concentrations and dispensed into 96-well microdilution trays at a final concentration of 0.016–16 μ g/ml for AmB, ITC; and 0.063–64 μ g/ml for FLU. All clinical isolates were cultured on malt extract agar (MEA, Difco, Detroit, MI, USA) at 35°C in dark and inoculum suspensions were prepared by harvesting the cell from 24 hours old cultures and were adjusted spectrophotometrically in saline to optical densities ranged 75-77% transmission. Final inoculum ranged from 2.5 $\times 10^3$ to 5 $\times 10^3$ CFU/ml as demonstrated by a quantitative colony count on Sabouraud's dextrose agar (SDA, Difco, Detroit, MI, USA). MIC values were determined visually after 24h at 35°C. The resistance breakpoints are fluconazole ≥ 8 ,

itraconazole ≥ 1.0 , amphotericin B ≥ 1.0 (8, 9).

Results

Forty-three patients (40.9%) had Candida infection in the present study. The most samples were obtained from nail clippings (39.5%), blood (13.9%), and thrush (11.6%). Age range of patients was between 18 days to 16 years (mean age; 5.2 years). Male to female sex ratio was 24/19. One patient had chronic mucocutaneous candidiasis (CMC). He was underwent bone marrow

transplantation. *Candida albicans* was the most common species isolated from patients (46.5%) followed by *C. parapsilosis* (18.6%) and *C. kefyr* (11.6%) (Figure1). Table I shows patients descriptions in details. MICs ranges for amphotericin B, fluc .onazole, and itraconazole were (0.025-0.75 $\mu\text{g/ml}$), (0.125-16 $\mu\text{g/ml}$), and (0.094-2 $\mu\text{g/ml}$), respectively (Table II).

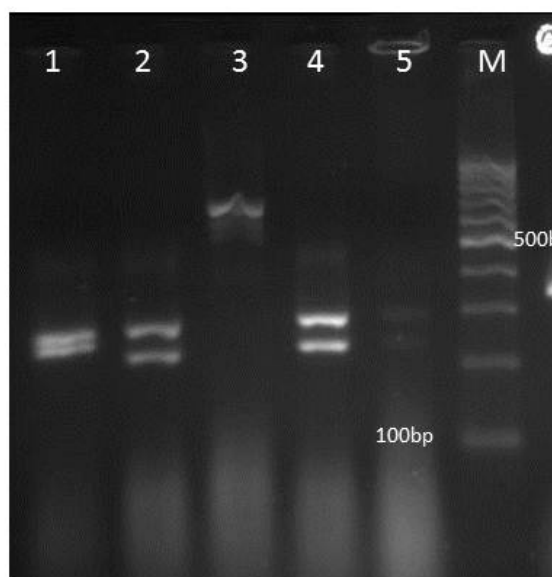


Figure1. Agarose gel electrophoresis of ITS-PCR products of *Candida* isolates after digestion with *MspII*. Lanes 1:*C.krusei*, lanes 2,4,5: *C. albicans*, lane 3: *C. kefyr*, and lanes M is 100 bp DNA size marker.

Table I: Details of patients with candidiasis in the present study.

No.	Sex	Age	Clinical location	Predisposing factors	Candida spp.
1	M	5	Nail	Diabetes	<i>C. krusei</i>
2	M	6 mon	Thrush	-	<i>C. albicans</i>
3	M	9	BAL*	-	<i>C. albicans</i>
4	F	12	Nail	Diabetes	<i>C. krusei</i>
5	F	6	Nail	-	<i>C. parapsilosis</i>
6	M	8	Nail	Use of antibiotic	<i>C. parapsilosis</i>
7	F	6	Nail	Nutrients deficiency	<i>C. kefyr</i>
8	F	6	Thrush	-	<i>C. albicans</i>
9	F	14	Groin	-	<i>C. albicans</i>
10	F	8 mon	Thrush	-	<i>C. albicans</i>
11	F	16	Nail	Diabetes	<i>C. parapsilosis</i>
12	M	10	Nail	Leukemia	<i>C. parapsilosis</i>
13	F	7	Nail	Nutrients deficiency	<i>C. parapsilosis</i>
14	M	7 mon	Thrush	-	<i>C. albicans</i>
15	M	10	Oropharynx+BAL	Lymphoma	<i>C. albicans</i>
16	F	6	BAL	Use of antibiotic	<i>C. albicans</i>
17	M	1.5	Blood	Leukemia	<i>C. albicans</i>
18	F	6	Eye discharge	-	<i>C. albicans</i>
19	M	3	Sore	Burning	<i>C. parapsilosis</i>
20	F	1	Nail	-	<i>C. tropicalis</i>
21	F	1	Blood	-	<i>C. albicans</i>
22	M	3	Nail	Diabetes	<i>C. kefyr</i>
23	M	14	Oropharynx	Nutrients deficiency	<i>C. krusei</i>
24	F	6	Head	BM transplantation	<i>C. albicans</i>
25	M	6	Blood	Use of catheter	<i>C. albicans</i>
26	M	4	Nail	-	<i>C. albicans</i>
27	M	8	Oropharynx	-	<i>C. kefyr</i>
28	M	4	Skin	Use of antibiotic	<i>C. kefyr</i>
29	F	14	Blood	Leukemia	<i>C. albicans</i>
30	F	2	Nail	Lymphoma	<i>C. albicans</i>
31	F	3	Nail	-	<i>C. krusei</i>
32	F	3	Nail	-	<i>C. parapsilosis</i>
33	M	2	Urine	Use of antibiotic	<i>C. tropicalis</i>
34	M	10	Urine	Diabetes	<i>C. tropicalis</i>
35	M	1	Urine	-	<i>C. tropicalis</i>
36	M	2	Nail	-	<i>C. kefyr</i>
37	M	2	Nail	-	<i>C. parapsilosis</i>
38	M	4	Nail	-	<i>C. guilliermondii</i>
39	F	1.5	Groin	-	<i>C. albicans</i>
40	M	18 days	Blood	Use of catheter	<i>C. albicans</i>
41	F	35 days	Blood	Use of catheter	<i>C. albicans</i>
42	M	6.5	Skin	-	<i>C. glabrata</i>
43	M	55 days	Thrush	-	<i>C. albicans</i>

Mon: month, BAL: Broncho-alveolar lavage, BM: Bone marrow

Table II: In vitro antifungal susceptibility testing of *Candida* spp. isolated from pediatrics.

No.	Candida spp. Candida spp.	AP MIC (µg/ml)	FL MIC (µg/ml)	IT MIC (µg/ml)
1	<i>C. krusei</i>	0.5	1.5	0.5
2	<i>C. albicans</i>	0.025	1	1
3	<i>C. albicans</i>	0.5	1	0.125
4	<i>C. krusei</i>	0.094	1.5	1
5	<i>C. parapsilosis</i>	0.025	0.5	0.25
6	<i>C. parapsilosis</i>	0.5	0.5	0.5
7	<i>C. kefyr</i>	0.19	0.25	0.094
8	<i>C. albicans</i>	0.094	0.5	0.25
9	<i>C. albicans</i>	0.125	1	0.25
10	<i>C. albicans</i>	0.047	16	2
11	<i>C. parapsilosis</i>	0.5	0.125	0.5
12	<i>C. parapsilosis</i>	0.094	0.5	0.094
13	<i>C. parapsilosis</i>	0.5	0.125	0.5
14	<i>C. albicans</i>	0.19	0.75	0.25
15	<i>C. albicans</i>	0.094	0.5	0.5
16	<i>C. albicans</i>	0.025	0.5	0.094
17	<i>C. albicans</i>	0.094	0.25	0.94
18	<i>C. albicans</i>	0.19	0.125	0.5
19	<i>C. parapsilosis</i>	0.094	1	0.25
20	<i>C. tropicalis</i>	0.5	1	1
21	<i>C. albicans</i>	0.025	0.5	0.5
22	<i>C. kefyr</i>	0.125	0.25	0.094
23	<i>C. krusei</i>	0.19	4	1
24	<i>C. albicans</i>	0.125	0.125	0.094
25	<i>C. albicans</i>	0.5	0.5	0.25
26	<i>C. albicans</i>	0.19	0.25	0.125
27	<i>C. kefyr</i>	0.75	0.75	0.094
28	<i>C. kefyr</i>	0.094	0.25	0.94
29	<i>C. albicans</i>	0.5	1.5	0.094
30	<i>C. albicans</i>	0.19	0.5	0.5
31	<i>C. krusei</i>	0.025	1	1
32	<i>C. parapsilosis</i>	0.5	0.5	0.5
33	<i>C. tropicalis</i>	0.025	0.125	0.25
34	<i>C. tropicalis</i>	0.047	1	0.094
35	<i>C. tropicalis</i>	0.5	0.5	0.5
36	<i>C. kefyr</i>	0.094	1	0.5
37	<i>C. parapsilosis</i>	0.025	0.125	0.094
38	<i>C. guilliermondii</i>	0.125	0.75	0.5
39	<i>C. albicans</i>	0.19	0.25	0.5
40	<i>C. albicans</i>	0.094	0.5	0.25
41	<i>C. albicans</i>	0.19	0.125	0.25
42	<i>C. glabrata</i>	0.19	0.5	0.5
43	<i>C. albicans</i>	0.19	0.125	0.5

AP: Amphotericin B, FL: Fluconazole, IT: Itraconazole.

Discussion

Several *Candida* species are colonized on the skin surfaces and mucosal layers of humans. Immunosuppressed patients are more susceptible to develop both superficial and life-threatening *Candida* infections (10). Candidiasis is also the most prevalent fungal infections in AIDS patients (10, 11). This group mainly infected to oropharyngeal candidiasis, which can lead to malnourishment and obstruct the absorption of medication. There was no any HIV+ patient, but 3 patients (7%) were diagnosed with oropharyngeal candidiasis and malnourishment. Although *C. albicans* is the most common species connected to candidiasis, the incidence of non-*albicans* *Candida* species is increasing. This alteration in epidemiology could be connected to prematurity, the severe illnesses or immunosuppression conditions, use of broad-spectrum antibiotics, and elderly (12). The prevalence of nail infections elevates with age, diabetes, nail trauma, circumferential circulation, long-time exposure to the pathogenic fungi, use of broad-spectrum antibiotics, corticosteroid therapy, and immune system disorders (13). *Candida* nail infections occur in patients with chronic mucocutaneous candidiasis, and are found more frequently in females than males (14), in agreement with the present study. Nail infection affect the middle finger due to contact with *Candida* strains that are in the intestine or vagina (14, 15). Middle finger was affected in all patients with nail infection in the present study with the exception of 6 patients. Tortorano et al., in 2006, reported that in European countries, more than half of the candidaemia cases were caused by *C. albicans*, followed by *C. glabrata* (14%) *C. parapsilosis* (14%), *C. tropicalis* (7%), and *C. krusei* (2%) (16). Ajenjo et al. also revealed that the prevalence of *C. albicans* has altered in Chile, and an accelerating

increase of non-*albicans* *Candida* infection has been noticed. They recognized *C. parapsilosis* as the most common species, followed by *C. tropicalis*, and *C. glabrata*. All isolates were susceptible to amphotericin B; however, 50% of the *C. glabrata* isolates were resistant to fluconazole (17), however, the etiologic agent of all candidemia cases in the present investigation was *Candida albicans*, and *in vitro* antifungal susceptibility pattern showed that all *C. albicans* isolated from bloodstream were susceptible to amphotericin B, and fluconazole. *C. parapsilosis* has appeared as a nosocomial fungal pathogen with clinical signs containing arthritis, endocarditis, endophthalmitis, peritonitis and fungaemia, usually connected to prosthetic devices or invasive procedures (18). In Spain, Canton et al., in 2011, showed that *C. Parapsilosis* is the second most frequently *Candida* spp. isolated from blood stream after *C. albicans* (18); whereas, no *C. parapsilosis* strain was isolated among candidemia cases in the present study. *Candidaemia* due to *C. tropicalis* has been connected to the malignancies, particularly in patients with neutropenia and leukemia (19), but in this investigation, the etiologic agent of candidemia in patients with leukemia (no. 17 and 29) was *C. albicans*. *Candidaemia* caused by *C. glabrata* has been described to be connected to the use of azoles like fluconazole (20). In the present investigation only one *C. glabrata* strain was isolated from skin lesions however, blood stream infection was not associated with *C. glabrata*. *Candida guilliermondii* was formerly unusual *Candida* spp., however, the prevalence of *C. guilliermondii* is increasing, too (20, 21). This investigation isolated a *Candida guilliermondii* (2.3%) from patient no. 38 with nail infection. *Candida* spp. causes candiduria in 22% of patients entered into the intensive care unit (ICU) (22),

nevertheless 3 patients (7%) had candiduria in the present study. The colonization of *Candida* species in the respiratory tract is usual in the patients receiving mechanical ventilation for periods of longer than 2 days. This happens because of haematogenous spread or pulmonary aspiration of the substances of fungal colonies of oropharyngeal origin (23). A patient with oropharyngeal candidosis was diagnosed in the present study (patient no. 15). Pathogenicity of *Candida* species depends on many virulence factors, such as biofilm formation, adherence ability to the host tissues and medical appliances like catheters, and secretion of some hydrolytic enzymes (24). Three out of 4 patients with candidemia used catheter as a predisposing factor of candidemia in the present investigation. It can connect to the biofilm formation on the catheters. Among the non-*albicans* *Candida* species, *C. parapsilosis* and *C. tropicalis* are usually susceptible to azoles; but, *C. tropicalis* is less susceptible to fluconazole than *C. albicans* (25, 26). It is in accordance with these findings because 2 out of 4 *C. tropicalis* were resistant to fluconazole. *C. glabrata* is intrinsically more resistant to antifungal drugs especially to fluconazole (21). *C. glabrata* strain (no. 42) was susceptible to all antifungal agents used in the present study. Seifi et al., (27) in 2013, reported 5.2% candiduria among children in Ahvaz, whereas 6.9% candiduric patients were diagnosed in the present study. In another study in Ilam (28), resistance rate of *Candida* strains isolated from children with oral candidosis and diaper dermatitis to fluconazole and itraconazole, was 43% and 34.2%, respectively, while 2.3% of clinical isolates were resistant to these antifungal drugs in the present study.

Conclusion

Considering the high incidence of *Candida* infections caused by non-*albicans* species, increasing of fatal infection like candidemia, excessive exposure to the antifungal agents, and the appearance of

antifungal resistance isolates, successful treatment of candidiasis is based on the early and precise identification of the *Candida* species and determination of antifungal susceptibility patterns of clinical isolates, as it was done in this study.

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Conflict of interest

The authors declare that they have no conflict of interest in this study.

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