

## RESEARCH ARTICLE

# Phenotype, genotype, and mating type determination in oral *Candida albicans* isolates from pediatric patients with neutropenia

Hadis Jafarian<sup>1,2</sup>  | Maral Gharaghani<sup>3</sup>  | Ali Amin Asnafi<sup>4</sup>  | Amir Kamal Hardani<sup>5</sup>  | Ali Zarei-Mahmoudabadi<sup>1,2</sup> 

<sup>1</sup>Department of Medical Mycology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

<sup>2</sup>Infectious and Tropical Diseases Research Center, Health Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

<sup>3</sup>Medicinal Plants Research Center, Yasuj University of Medical Sciences, Yasuj, Iran

<sup>4</sup>Thalassemia and Hemoglobinopathy Research Center, Health Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

<sup>5</sup>Department of Pediatrics, School of Medicine, Abuzar Children Hospital, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

## Correspondence

Ali Zarei-Mahmoudabadi, Infectious and Tropical Diseases Research Center, Health Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.

Email: [zareid40@hotmail.com](mailto:zareid40@hotmail.com)

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## Abstract

**Background:** The most frequent species of *Candida* to infect and colonize patients with neutropenia is still *Candida albicans*. This study aimed to provide detailed information on the phenotype, genotype, and mating type of oral *C. albicans* isolated from neutropenic pediatric patients, and to investigate how these characteristics are related.

**Methods:** Two hundred fifty-four oral samples from patients under 18 years old with neutropenia and malignancies were collected from January to October 2021. Samples were cultured on CHROMagar *Candida*. Isolates of *C. albicans* were identified with the germ tube test, chlamyospore production on cornmeal agar, and PCR-RFLP. Genotyping of *C. albicans* isolates was carried out by amplifying the 25S rDNA gene with specific CAINT-L and CA-INT-R primers. *MTLa1* and *MTL $\alpha$ 1* primers were used to identify each mating type. Yeast peptone dextrose supplemented with phloxine B was used to identify different phenotypes.

**Results:** Ninety-two (36%) patients were positive for *C. albicans*. The mean age of patients was 7.85. Fifty-three (58.9%) isolates demonstrated type A, 15 (16.7%) type B, 15 (16.7%) types D/E, and 7 (7.7%) type C. Three isolates each (3.3%) were homozygous for *MTLa* or homozygous for *MTL $\alpha$* . All of the *MTL*-homozygous isolates were genotype A. There was a significant correlation between patients' underlying disease and genotype ( $p = 0.036$ ). There was a significant correlation between mating type and genotype ( $p = 0.000$ ).

**Conclusion:** Most of the isolates exhibited a white phenotype, noted in the literature as the most virulent. Moreover, heterozygous strains were frequent and may play a role in *Candida* colonization.

## KEYWORDS

*Candida albicans*, genotype, neutropenic, pediatric

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## 1 | INTRODUCTION

Children with neutropenia and malignancies are at an increased risk of developing oral candidiasis, which may lead to systemic infections.<sup>1</sup> *Candida albicans* is still the most common *Candida* species infecting and colonizing neutropenic patients,<sup>2,3</sup> and has pathogenic potential for disseminated infection in immunocompetent hosts.<sup>4</sup> As part of the normal human flora it is generally harmless, but in immunosuppressed individuals it can cause serious diseases.<sup>5</sup> Radiation, bone marrow failure, chemotherapy, and hematopoietic cell replacement by malignant cells in the bone marrow are potential causes of neutropenia.<sup>6,7</sup> In patients with acute neutropenia and leukemia, the digestive tract serves as the primary entry point for *Candida* species. The natural anatomical barriers may be damaged, allowing *Candida* to enter the bloodstream. In these persons, *Candida* infections can manifest as esophagitis, oropharyngeal candidiasis, candidemia, and acute or chronic disseminated candidiasis.<sup>8–10</sup>

There are several methods for typing *C. albicans* such as microsatellite length polymorphism<sup>11</sup> and multilocus sequence typing.<sup>12</sup> Another approach is based on 25S ribosomal DNA (rDNA). This method is based on sequence variation of the transcribed spacer locus (25S rRNA gene) described by McCullough et al., which can be used to categorize different strains into five types (A, B, C, D, and E).<sup>13</sup> Phenotypic switching and biofilm formation are one of the virulence factors of *C. albicans*.<sup>14,15</sup> The white-opaque switch affects the morphology, configuration of gene expression, physiology, and virulence of *C. albicans*.<sup>16</sup> Correspondingly, it has been confirmed that a variety of virulence features differ between cells in the two phases.<sup>17,18</sup> It was reported that strains causing deep tissue fungal infections switch at higher frequencies, on average, than strains causing superficial infections.<sup>14</sup>

Most natural *C. albicans* isolates are heterozygous for the mating-type locus. Heterozygous strains are not able to mate.<sup>19–22</sup> Homozygous strains can undergo mating and switch from the white to the opaque phenotype.<sup>19–21</sup> Earlier studies suggested that *MTL*-homozygous strains may take part in colonization.<sup>22,23</sup> One study demonstrated that *C. albicans* strains capable of mating exist naturally in patients, and the authors proposed that mating may play a role in the appearance of diversity in this lethal pathogen.<sup>20</sup> Although our understanding of how these features contribute to infection has increased significantly in recent years, further studies are necessary in this area to shed light on some unanswered questions. Is there any relationship between phenotype switching and genotype, or between genotype and mating type in clinical isolates? The present study aimed to provide detailed information on the phenotype, genotype, and mating type of oral *C. albicans* isolated from neutropenic pediatric patients with neutropenia, and to investigate how these characteristics are related.

## 2 | MATERIALS AND METHODS

The Ethics Committee of Ahvaz Jundishapur University of Medical Sciences approved this project under reference no. IR.AJUMS.

MEDICINE.REC. 1399.037. Moreover, all researchers involved in the study complied with the current version of the World Medical Association's Declaration of Helsinki in accordance with the January 1997 Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95).

### 2.1 | Samples and identification

Two hundred fifty-four oral samples from patients under 18 years old with neutropenia and malignancies were collected from January to October 2021. Samples were cultured on CHROMagar *Candida* (CHROMagar, France), and positive cultures were purified on Sabouraud dextrose agar (Liofilchem). *Candida albicans* isolates were identified with the germ tube test, chlamyospore production on cornmeal agar (HIMEDIA, India) supplemented with Tween 80 (Merck, Germany), growth at 43°C and PCR-RFLP according to Mirhendi et al.<sup>24</sup> using ITS1 and ITS4 primers.<sup>25</sup>

DNA was extracted according to Look et al.<sup>26</sup> One or two fresh purified colonies were transferred to a tube containing 100 µl 0.2 M lithium acetate (Central Drug House) and 1% SDS (CinnaGen). Each tube was incubated for 5 min in a 70°C water bath. Then absolute ethanol was added and each tube was gently vortexed. The tubes were centrifuged for 2 min at 18,000 g, after which the supernatants were discarded. Then 300 µl 70% ethanol was added to the pellet and the tubes were centrifuged at 18,000g for 2 min. The supernatant was removed, and tubes were air-dried at room temperature. Then 100 µl sterile distilled water was added to each tube, and the tubes were gently vortexed and centrifuged for 2 min at 18,000g. Lastly, the supernatant containing DNA was transferred to a new tube and stored at 20°C.

### 2.2 | ABC genotyping

Genotyping of *C. albicans* isolates was carried out by amplifying the 25S rDNA gene with specific primers CAINT-L (5'-ATAAGGGAAGT CGGCAAATAGATCCGTAA-3') and CA-INT-R (5'-CCTTGGCTGT GGTTCGCTAGATAGTAGAT-3').<sup>13</sup> Briefly, Taq DNA Polymerase 2x Master Mix RED with 1.5 mM MgCl<sub>2</sub> (Ampliqon), 0.5 µM of each primer, and 2 µl genomic DNA in a total volume of 25 µl. PCR products were amplified under the following conditions: 97°C for 7 min, 35 cycles at 94°C for 30s, 60°C for 30s, 72°C for 40s, and 72°C for 5 min as the final extension. The PCR products were visualized on 1.5% agarose gel. A 450bp band represented type A, 840bp type B, 450 and 840bp type C, 1040bp type D, and 1080bp type E. Types D and E correspond to *C. dubliniensis*.

### 2.3 | Mating type

*MTLa1* (forward: 5'-TAAGAATGAAGACAACGAGG-3' and reverse: 5'-CGTGTTTTCTGCTATCAATTCC-3') and *MTLα1* (forward:

**TABLE 1** Phenotypic characteristics of each genotype

Genotype	Color in CHROMagar	Germ tube test	Chlamydospore production	Growth at 43°C
A (53/58.9%)	Green	Positive	Positive	Positive
B (15/16.7%)	Green	Positive	Positive	Positive
C (7/7.7%)	Green	Positive	Positive	Positive
D/E (15/16.7%)	Green	Positive	Positive	Negative



**FIGURE 1** Genotyping of *Candida albicans* isolates. Lane 1: type A (450 bp), lane 2: type B (840 bp), lane 3: type C (450 and 840 bp), lane 4: type D/E (1040 bp)

5'-TACATTCTGGTCGCGATGCTC and reverse: 5'-GTAATCCAAAGCCTCGCATAA-3') primers were used to identify the mating type of each isolate.<sup>27</sup> Multiplex PCR assays were carried out with 1× Taq DNA Polymerase 2× Master Mix RED 1.5 mM MgCl<sub>2</sub> (Ampliqon, Denmark), 0.5 μM of each primer, and 3 μl genomic DNA in a total volume of 25 μl. PCR products were amplified under the following conditions: 94°C for 10 min, 30 cycles at 94°C for 60s, 57°C for 45s, 72°C for 45s, and 72°C for 7 min as the final extension. The PCR products were visualized on 1.5% agarose gel. A 535 bp band represented type α, and a 423 bp band represented type a. Heterozygous strains exhibited both bands.

## 2.4 | Phenotyping on phloxine B medium

Yeast peptone dextrose (YPD) agar containing 20 g glucose (PanReac), 10 g yeast extract (Merck), 20 g peptone (Difco), and 10 g agar (Conda) supplemented with 5 mg/L phloxine B was used to determine the phenotype of each isolate (Merck).<sup>28</sup> Fresh yeast cells were diluted in sterile distilled water and spread at a density of 30–50 CFU/ml on YPD agar plates, which were then incubated at 30°C for 4–5 days.

## 2.5 | Statistical analysis

The data were analyzed with SPSS software version.<sup>22</sup> Descriptive statistics and chi-squared tests were used to summarize the results. The findings were considered statistically significant at the  $p < 0.05$  level.

## 3 | RESULTS

A total of 254 oral samples were collected, and 92 (36%) patients were positive for *C. albicans*. Also, 52 non-*Candida albicans* isolates were identified including 10 *C. glabrata*, 10 *C. parapsilosis* complex, 8 *C. kefyr*, 7 *C. tropicalis*, 7 *C. krusei* and 10 isolates of other species. The mean age of patients was 7.85 years, and the age range was 1–18 years. Also, 56.5% of patients were male and 40% were female. The most common underlying conditions among patients with positive culture for *C. albicans* were acute lymphoblastic leukemia (30.93%), brain tumor (8.25%), Ewing sarcoma (6.19%), anemia (6.19%), rhabdomyosarcoma (5.15%), immune thrombocytopenia (ITP) (4.12%), Burkitt lymphoma (4.12%), Wilms tumor (4.12%), acute myeloid leukemia (4.12%), neuroblastoma (3.09%), lymphoma (3.09%), Hodgkin's lymphoma (3.09%), sarcoma (3.09%), tumor (3.09%), medulloblastoma (2.07%), abdominal mass (2.07%), osteosarcoma (2.07%), germcell tumor (1.03%), lymphoblastic lymphoma (1.03%), thrombocytopenia (1.03%), pancytopenia (1.03%) and paroxysmal nocturnal hemoglobinuria (PNH) (1.03%).

Ninety-two positive cultures for *C. albicans* complex were detected. Fifty-three isolates demonstrated type A, 15 type B, 15 types D/E, and 7 type C (Table 1 and Figure 1). In two isolates amplification was not successful, and these samples were excluded from the study. The frequency of each phenotype according to color (Figure 2) and shape (Figure 3) among different genotypes is shown in Tables 2 and 3. Most of the *C. albicans* isolates were MTL-heterozygous (69, 92%). Three isolates (4%) were homozygous for MTLα and 3 isolates (4%) were homozygous for MTLa. All MTL-homozygous isolates were genotype A. There was no significant correlation between the mating type and phenotype ( $p = 0.310$ ). Also, there was no significant correlation between patients' underlying disease and mating type ( $p = 0.452$ ). There was a significant correlation between patients' underlying disease and genotype ( $p = 0.036$ ). There was a significant correlation between mating type and genotype ( $p = 0.000$ ).

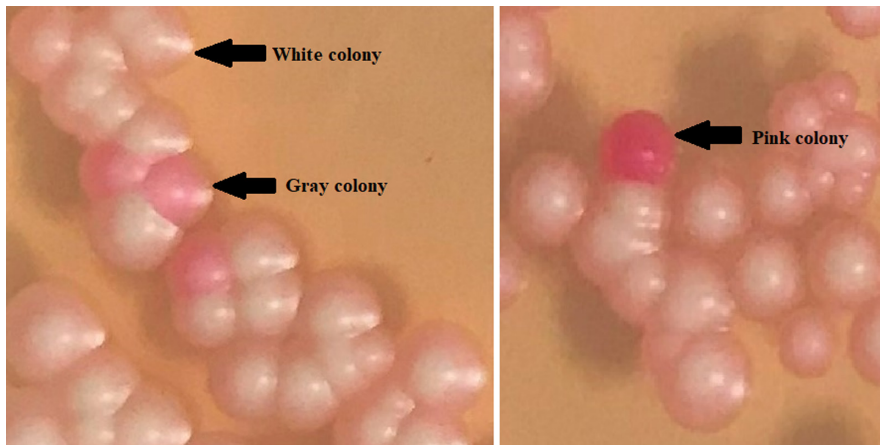


FIGURE 2 Different phenotypes observed according to colony color

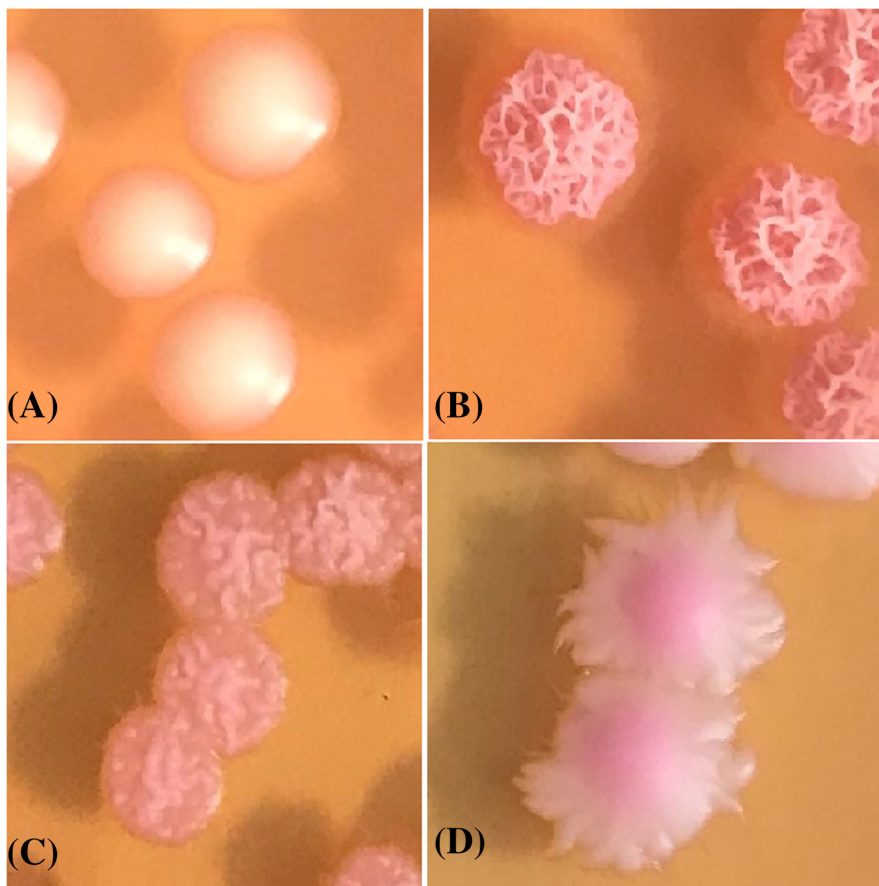


FIGURE 3 Different phenotypes observed according to colony shape. (A) Smooth colonies, (B) Irregular colonies, (C) Stippled colonies, (D) Fuzzy colonies

#### 4 | DISCUSSION

Children are more likely than adults to develop oral difficulties such as dry mouth, bleeding, oral mucositis, and infections due to chemotherapy and radiotherapy.<sup>29</sup> Oral health is severely impaired by cancer and cancer therapy. Oral candidiasis is a problem because it can lead to systemic infection, which may be life-threatening in pediatric patients.<sup>30,31</sup> Preventative prophylaxis before starting chemotherapy can help to reduce the incidence of oral candidiasis in children with cancer. In the present study, we examined 254 oral swabs and 36% of them were positive for *C. albicans*. Badiie et al. examined 118 patients and found a prevalence of *C. albicans* in oral samples of 44.9%.<sup>3</sup>

González et al. reported 69.35% of their patients were positive for oral candidiasis with *C. albicans*.<sup>32</sup> Also, Aslani et al. isolated 162 yeasts and yeast-like fungi from oral samples of adult patients with cancer, and *C. albicans* was reported as the most frequent isolate (50.6%).<sup>33</sup> *Candida albicans* is still the most common yeast isolated in the oral cavity of patients with cancer, and may lead to more serious infections.

Kinifar and coworkers studied *C. albicans* strains isolated from healthy individuals. In consonance with our results, genotype A was the frequent genotype (41.7%)<sup>34</sup> but the frequency rate was higher in children with neutropenia (57.6%). These results were also in line with our previous study in patients with esophageal candidiasis.<sup>28</sup> However, genotype A was the most prevalent type in oral isolates

**TABLE 2** Incidence of each phenotype according to colony color in different genotypes

Colony color	ABC genotype				Total
	A	B	C	D/E	
White & Gray	24	6	3	4	37
Gray	8	4	1	6	19
White	10	2	2	1	15
White & Gray & Pink	6	2	1	2	11
White & Pink	3	1	0	0	4
Pink & Gray	2	0	0	2	4
Total	53	15	7	15	90

**TABLE 3** Incidence of each phenotype according to colony shape in different genotypes

Colony shape	ABC genotype				Total
	A	B	C	D/E	
Smooth	32	10	4	11	57
Stippled	0	0	0	1	1
Fuzzy	0	0	0	1	1
Smooth & Irregular	21	5	3	2	31
Total	53	15	7	15	90

or strains related to the gastrointestinal tract. Regarding other genotypes, Kinifar et al. found higher frequencies of genotypes C (34%) and B (20.4%).<sup>34</sup> In contrast, the frequency of genotype D was higher in the present study (16.7%). Sardi et al.<sup>35</sup> reported genotype B (51.6%) as the major genotype, and Gharaghani et al. reported genotype C (83.5%) as the predominant genotype in vulvovaginal samples.<sup>36</sup> Also, about 16% of isolates in the present study were genotype B. Chaves et al.<sup>37</sup> stated that *C. albicans* isolates with genotype B have a high propensity to cause invasive infections. These infections may increase the mortality rate in immunocompromised children. Rosca et al. studied isolates from various clinical samples and did not find any significant correlation between the genotype and the site of infection.<sup>38</sup> We observed a significant correlation between patients' underlying disease and genotype ( $p = 0.036$ ). This finding may be related to the specific treatment procedure or the disease, but due to the low frequency of some underlying diseases and genotypes, additional studies are required.

*Candida albicans* is one of the most adaptable organisms, with the ability to switch between morphological phenotypes in response to environmental stimuli, which is critical for its survival as a commensal or pathogenic organism.<sup>39,40</sup> It is a diploid organism. Homozygosity and heterozygosity were investigated in genes like hyphal wall protein 1 gene locus (*hwp1*) previously and no significant statistical differences in virulence factors between the homozygous and heterozygous strains were reported.<sup>41</sup> In the present study, 70% of isolates exhibited the white phenotype (pure or in combination

with other colonies), which may be more virulent according to research by Tao et al.<sup>42</sup> They reported white colonies to be more virulent than gray and opaque phenotypes. Moreover, switching between the white phenotype and the opaque phenotype is required for the yeast to mate. Previously, it was assumed that only *MTL*-homozygous strains could switch to the opaque phenotype. Xie et al.<sup>22</sup> demonstrated that about one-third of heterozygous strains isolated from hosts underwent white-opaque switching at low frequencies when cultured on medium containing N-acetylglucosamine (25°C and 5% CO<sub>2</sub>).<sup>22</sup> These strains play an important role in colonization. Most of the strains isolated in the present study were heterozygous, although it should be recalled that harmless colonized isolates can turn into dangerous pathogens in susceptible hosts. In this connection, Kvaal et al.<sup>23</sup> showed that homozygous strains that switched to the opaque form can affect the ability to colonize skin; this change may play a role in transmission from patients' skin to healthcare workers, and subsequently to immunocompromised patients. Norma et al.<sup>43</sup> reported that mating rarely occurred during oropharyngeal candidiasis in immunosuppressed mice. Ramirez-Zavala et al.<sup>44</sup> found that white-opaque switching can be induced in the mouse gastrointestinal tract. In a related outcome, we found a significant correlation between mating type and genotype ( $p = 0.000$ ). This relationship may be due to the lower frequency of some types, and further research is thus needed.

In summary, we observed a significant correlation between patients' underlying disease and *C. albicans* genotype. A higher frequency of genotype D was observed in pediatric patients with neutropenia. Most of the isolates exhibited the white phenotype, which has been suggested to be the most virulent phenotype in earlier research. Moreover, heterozygous strains were highly frequent, and may play a role in *Candida* colonization and transmission.

We note important limitations in this study arising from the Covid-19 pandemic. Nonetheless, we recommend further research into the relationships between mating type and virulence factors in *C. albicans* isolates.

#### AUTHOR CONTRIBUTIONS

AZM, AAA, AKH: Study design, supervision, data interpretation, and manuscript editing. HJ: Experimentation, data collection, analysis, literature search, and manuscript writing. MG: Experimentation, data collection, analysis.

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#### CONFLICT OF INTEREST

The authors state that there is no competitive concern of any nature with this manuscript.



## DATA AVAILABILITY STATEMENT

All derived data supporting the findings of this study are available from the corresponding author Ali Zarei Mahmoudabadi on request.

## INFORMED CONSENT

Informed consent was obtained from all individual participants included in the study.

## ORCID

Hadis Jafarian  <https://orcid.org/0000-0002-8959-6482>

Maral Gharaghani  <https://orcid.org/0000-0002-0144-3407>

Ali Amin Asnafi  <https://orcid.org/0000-0002-0730-4360>

Amir Kamal Hardani  <https://orcid.org/0000-0002-7734-4181>

Ali Zarei-Mahmoudabadi  <https://orcid.org/0000-0002-5347-2082>

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