

## Major Article

# Distribution of clinical isolates of *Candida* spp. and antifungal susceptibility of high biofilm-forming *Candida* isolates

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### Abstract

**Introduction:** The increase in the incidence of fungal infections, especially those caused by *Candida albicans* and other *Candida* species, necessitates the understanding and treatment of *Candida*-associated infections. In this study, we aimed to investigate the identification, distribution, and biofilm formation ability of different clinical *Candida* isolates and evaluate the distribution and antifungal susceptibilities of high biofilm-forming (HBF) *Candida* isolates. **Methods:** For identification, carbohydrate fermentation, carbohydrate assimilation, and ChromAgar tests were used. Biofilm formation was assessed using crystal violet binding assay, while the susceptibility to antifungal agents was determined using ATB™ Fungus 3 test kits. **Results:** The majority of *Candida* species were *C. parapsilosis* (31.3%; 31/99) and *C. tropicalis* (30.3%; 30/99). *C. tropicalis* was found to be the most frequently isolated species among all HBF *Candida* species. HBF *Candida* isolates were more frequently isolated from vaginal swab (35.7%; 10/28), tracheal aspirate (17.9%; 5/28), and urine (17.9%; 5/28). The majority of tested isolates were resistant to itraconazole and voriconazole, whereas no isolate was deemed resistant to 5-flucytosine. **Conclusions:** *C. tropicalis* displays the highest biofilm formation ability among all the *Candida* species evaluated, and HBF *Candida* isolates were more frequently seen in vaginal swab, tracheal aspirate, and urine samples. Our findings revealed that 5-flucytosine is the most efficient antifungal agent against HBF *Candida* isolates.

**Keywords:** Antifungal resistance. Biofilm formation. *Candida albicans*. Non-*Candida albicans*. *Candida* Species. 5-Flucytosine

### INTRODUCTION

*Candida* species are natural colonizers of gastrointestinal and urogenital tracts and known to reside as commensals in the oral and conjunctival flora of the healthy human body<sup>1</sup>. These organisms are known as opportunistic pathogens that may cause various infections ranging from oral candidiasis and esophagitis to hospital-acquired blood stream infections<sup>2,4</sup>. Although *Candida albicans* has been reported as the most predominant *Candida* species that frequently causes invasive fungal infections, a significant increase in non-*C. albicans* *Candida* (NCAC) species such as *Candida glabrata*, *Candida krusei*, *Candida tropicalis*, and *Candida parapsilosis* in human candidiasis has also been indicated over the last decade<sup>2,4,5</sup>. The increase in the occurrences of all NCAC species as pathogens has led to improvements in diagnostic methods that can sensitively differentiate between NCAC and *C. albicans*<sup>5</sup>.

On the other hand, the widespread use of a broad range of medical implant devices and an increase in patients that receive immunosuppressive therapy have led to the colonization of different *Candida* species and various *Candida* infections<sup>2,6</sup>. Biofilm formation is one of the most important reasons involved in the transformation of *Candida* species into important human pathogens<sup>6</sup>. Biofilm formation is responsible for many problems, as it avoids penetration and diffusion of various antimicrobial agents, causes generation of biofilm cells that have physiological and metabolic alterations, and provides a suitable environment for horizontal gene transfer mechanisms, which play an important role in antimicrobial resistance<sup>7,8</sup>. As biofilm environments are suitable for the acquisition of new traits via horizontal gene transfer<sup>9</sup>, investigation of the antifungal resistance of *Candida* isolates with biofilm formation ability and determination of effective antifungal agents against these isolates are necessary to prevent biofilm-associated *Candida* infections. In this study, we aimed to identify different clinical *Candida* isolates, determine their biofilm formation ability, and investigate the susceptibility of high biofilm-forming (HBF) *Candida* isolates to antifungal agents.

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## METHODS

### Microorganisms

We evaluated 99 clinical *Candida* isolates that were randomly collected from patients treated at two different hospitals in Ankara, Turkey, between July 2005 and March 2014. Collected isolates were inoculated into the brain heart infusion (BHI) broth (Lab M Ltd, Lancashire, UK) media supplemented with 10% glycerol and stored at -20°C for use in further experiments.

### Identification tests

Colony morphologies and microscopic images of collected isolates were examined. By visual inspections, cells and colonies suspected to be *Candida* were subjected to carbohydrate fermentation, carbohydrate assimilation, and ChromAgar tests.

### Carbohydrate fermentation tests

Carbohydrate fermentation tests were performed as per the method described by Bhavan<sup>10</sup>, with some modifications. Briefly, nutrient broth media supplemented with 1% (v/v) bromothymol blue as a pH indicator and carbohydrates such as glucose, galactose, lactose, maltose, and sucrose were separately prepared. A total of 10µL of each *Candida* isolate suspended in McFarland 0.5 standard in 5mL of saline buffer was added into 96 wells containing 100µL of different carbohydrate media. The plates were incubated at 37°C for 48h. Fermentation of any carbohydrate was considered as positive upon the change in the color of bromothymol blue to yellow. A total of 99 *Candida* isolates were identified according to their positive/negative carbohydrate fermentation test results, as presented in **Table 1**<sup>4,11-15</sup>.

### Carbohydrate assimilation tests

Carbohydrate assimilation tests were carried out as per the method described by Marinho et al.<sup>4</sup>, with some modifications. Briefly, 2% (w/v) carbohydrate solutions of glucose, galactose, lactose, maltose, sucrose, xylose, and raffinose were separately prepared and deposited onto sterile blotting paper discs prepared from eight layers of Whatman No. 1 filter paper. Each *Candida* isolate suspended in McFarland 0.5 standard in 5mL of saline buffer was inoculated onto 1% yeast nitrogen base (YNB)

agar medium (Difco™). The sterile carbohydrate discs were placed onto the agar plates and the plates were incubated at 37°C for 48h. Assimilation of any carbohydrate was considered as positive with a presence of a growth zone around the carbohydrate disc. A total of 99 *Candida* isolates were identified according to their positive/negative carbohydrate assimilation test results, as presented in **Table 1**<sup>4,11-15</sup>.

### Growths on chromagar media

Each *Candida* isolate was inoculated into CHROMagar™ *Candida* medium; (CAC, Becton Dickinson, Heidelberg, Germany), which is designed to identify different *Candida* species based on their colony colors and morphologies. All plates were incubated at 37°C for 48h and visually observed after incubation. A total of 99 *Candida* isolates were identified according to their colony morphologies on CHROMagar™ *Candida* medium. In CHROMagar™ *Candida* medium, smooth colonies that appear light to medium green were considered as *C. albicans*; while dark blue to metallic blue smooth colonies were considered as *C. tropicalis*. Pink colonies with a whitish rough border were deemed as *C. krusei*, whereas pink-lavender smooth colonies were considered as *C. glabrata*. In addition, pink-salmon smooth colonies were deemed as *Candida kefir*, while white-pale pink smooth colonies were considered as *C. parapsilosis*<sup>4,11,15</sup>.

### 18S ribosomal RNA gene sequence analysis

Within 99 *Candida* isolates, *Candida* isolates that could not be identified by the identification tests used in this study were identified by 18S ribosomal ribonucleic acid (rRNA) gene sequence analysis (RefGen Biotechnology Co. Ltd., Ankara, Turkey).

### Biofilm formation on 24-well polystyrene plates

Biofilm formation abilities of 99 different *Candida* isolates were determined by crystal violet binding assay described by O'Toole<sup>16</sup>, with some modifications. Briefly, single yeast colonies were picked-up from BHI agar plate and inoculated into 10-mL BHI broth medium and incubated at 37°C overnight. The overnight culture was 1:100 diluted into fresh BHI medium and the wells of a polystyrene plate were filled with 1mL of the diluted inoculum. The plates were incubated for 48h at 37°C.

**TABLE 1:** Carbohydrate fermentation and carbohydrate assimilation test results of different *Candida* species.

Carbohydrate fermentation					Carbohydrate assimilation						<i>Candida</i> species	
Gl	Ga	S	M	L	Gl	Ga	L	M	X	R		S
+	+/-	-	+	-	+	+	-	+	+/-	-	+/-	<i>C. albicans</i>
+	+	+/-	+	-	+	+	-	+	+	-	+/-	<i>C. tropicalis</i>
+	-	-	-	-	+	-	-	+/-	-	-	-	<i>C. glabrata</i>
+	+/-	-	-	-	+	+	-	+	+	-	+	<i>C. parapsilosis</i>
+	+	+	-	+	+	+	+	-	+/-	+	+	<i>C. kefir</i>
+	-	+/-	-	-	+	-	-	-	+/-	-	-	<i>C. krusei</i>

Gl: glucose; Ga: galactose; L: lactose; M: maltose; R: raffinose; S: sucrose; X: xylose.

After incubation, the medium was gently removed and the wells were gently washed with sterile distilled water. After allowing wells to dry, each well was stained with 1% (w/v) crystal violet (Merck)/sterile distilled water for 45 min at 25°C. Excess of crystal violet was removed by sterile distilled water and the bound crystal violet in each well was solubilized by adding 1mL of ethanol (96.6%) solution. Solubilized crystal violet from each well was read by a spectrophotometer (Shimadzu UV - 1700, Kyoto, Japan) at 560nm wavelength. According to biofilm formations, *Candida* isolates were classified into four categories as follows:

- $0 \leq OD < 0.4$ : non biofilm former (NBF)
- $0.4 \leq OD < 0.8$ : low biofilm former (LBF)
- $0.8 \leq OD < 1.2$ : intermediate biofilm former (IBF)
- $OD \geq 1.2$ : high biofilm former (HBF)

The experiment was performed in triplicates.

### Antifungal susceptibility tests

Antifungal susceptibilities of HBF *Candida* isolates [optical density (OD)  $\geq 1.60$ ] were determined by ATB™ Fungus 3 test kits (BioMérieux®, France). Antifungal susceptibilities of 25 HBF *Candida* isolates against 5-flucytosine, fluconazole, itraconazole, and voriconazole were evaluated. Briefly, all isolates were inoculated onto sabouraud dextrose agar (SDA) and incubated at 37°C for 48h. After incubation, each *Candida* isolate was suspended in saline solution, and yeast cells corresponding to a 2.0 McFarland standard were added into ATB F2 medium (yeast nitrogen base 6.7g; glucose 6.5g; asparagine 1.5g; disodium phosphate 2.5g; trisodium citrate 2.5g; potassium nitrate 5.5g; demineralized water 1,000mL; pH: 6.5-6.8). ATB F2 media with different *Candida* isolates were transferred into antifungal test strips and all the test strips were incubated at 37°C for 48h. After incubation, minimum inhibitory concentrations (MICs) of antifungal agents were visually determined and all the isolates were classified as resistant (R), intermediate (I), or sensitive (S) according to the MIC standards constituted by the Clinical and Laboratory Standards Institute (CLSI) (M27-A3, 2008)<sup>17</sup>. Breakpoints (mg/L) for *Candida* spp. were as follows:

	Sensitive	Intermediate	Resistant
5-Flucytosine	$\leq 4$	8 - 16	$\geq 32$
Fluconazole	$\leq 8$	16 - 32	$\geq 64$
Itraconazole	$\leq 0.125$	0.25 - 0.5	$\geq 1$
Voriconazole	$\leq 1$	2	$\geq 4$

### Statistical analysis

Chi-square analysis was applied to estimate differences between the effects of four different antifungal agents. Bonferroni *post-hoc* test was used to evaluate antifungal susceptibility and agents with more/less significant effects were estimated. The significance level was set at 5% and the difference between the effects of each antifungal agent was considered as significant when  $p$ -value  $< 0.05$ . Statistical analysis was performed using Statistical Package for Social Sciences (SPSS) 20.0 Software (IBM Corp, New York, USA).

## RESULTS

According to all phenotypic identification tests, six different *Candida* species, namely, *C. kefyr* ( $n = 1$ ), *C. glabrata* ( $n = 8$ ), *C. albicans* ( $n = 13$ ), *C. krusei* ( $n = 15$ ), *C. tropicalis* ( $n = 30$ ), and *C. parapsilosis* ( $n = 31$ ) were identified in this study. One of the *Candida* isolates could not be clearly identified by phenotypical methods and was thought to be *C. tropicalis* or *C. krusei*. The results of 18S ribosomal RNA gene sequence analysis identified this strain as *Candida orthopsilosis*.

The frequencies of different *Candida* species isolated in this study show that most of the *Candida* species were *C. parapsilosis* (31.3%;  $n = 31$ ) and *C. tropicalis* (30.3%;  $n = 30$ ), followed by *C. krusei* (15.2%;  $n = 15$ ), *C. albicans* (13.1%;  $n = 13$ ), *C. glabrata* (8.1%;  $n = 8$ ), *C. kefyr* (1%;  $n = 1$ ), and *C. orthopsilosis* (1%;  $n = 1$ ). Furthermore, the majority of *Candida* isolates were observed to be isolated from vaginal swab (49.5%;  $n = 49$ ), followed by specimens of tracheal aspirate (10.1%;  $n = 10$ ), blood (9.1%;  $n = 9$ ), sputum (9.1%;  $n = 9$ ), urine (8.1%;  $n = 8$ ), bronchoscopic culture (5.1%;  $n = 5$ ), wound (3%;  $n = 3$ ), bronchial lavage (1%;  $n = 1$ ), thoracentesis (1%;  $n = 1$ ), eye (1%;  $n = 1$ ), synovial fluid (1%;  $n = 1$ ), and catheter (1%;  $n = 1$ ). The examination of the distribution of different *Candida* species in different clinical specimens revealed *C. tropicalis* as the most frequent *Candida* species isolated from tracheal aspirate and urine specimens, while most of *Candida* isolates obtained from blood specimen were identified as *C. albicans* (Figure 1). *C. glabrata* was the only *Candida* species isolated from synovial fluid and thoracentesis specimens (Figure 1). However, all isolates obtained from catheter and bronchial lavage were *C. tropicalis* and all isolates isolated from wound and eye specimens were *C. parapsilosis* (Figure 1). Among all HBF *Candida* species such as *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, and *C. orthopsilosis*, *C. tropicalis* was found to be the most frequent species (23.2%;  $n = 23$ ) (Table 2). However, all *C. krusei* isolates were found as NBF (Table 2). HBF *Candida* isolates were obtained from clinical samples such as vaginal swab, tracheal aspirate, urine, catheter, sputum, bronchial lavage, bronchoscopic culture, and eye specimens (Figure 2). We evaluated the distribution of all HBF *Candida* isolates ( $n = 28$ ) in different clinical materials and found vaginal swab as the most frequent clinical material for HBF *Candida* isolate isolation (35.7%;  $n = 10$ ) (Figure 2). Furthermore, frequencies of HBF *Candida* isolates obtained from tracheal aspirate (17.9%;  $n = 5$ ), urine (17.9%;  $n = 5$ ), and sputum (14.3%  $n = 4$ ) were higher than those of HBF *Candida* isolates from catheter (3.6%;  $n = 1$ ), bronchial lavage (3.6%;  $n = 1$ ), bronchoscopic culture (3.6%;  $n = 1$ ), and eye (3.6%;  $n = 1$ ) specimens (Figure 2). The susceptibilities of 25 HBF *Candida* isolates to 5-flucytosine, fluconazole, itraconazole, and voriconazole were evaluated and all the agents were found to exhibit different effects (Chi-square test,  $p$ -value  $< 0.05$ ). Most isolates were resistant to itraconazole and voriconazole, while all were deemed sensitive to the effect of 5-flucytosine (Figure 3). Among all the antifungal agents used in this study, 5-flucytosine was efficient against HBF *Candida* isolates (Bonferroni *post-hoc* test,  $p$ -value  $< 0.00$ ) (Figure 3). Therefore, 5-flucytosine showed higher *in*

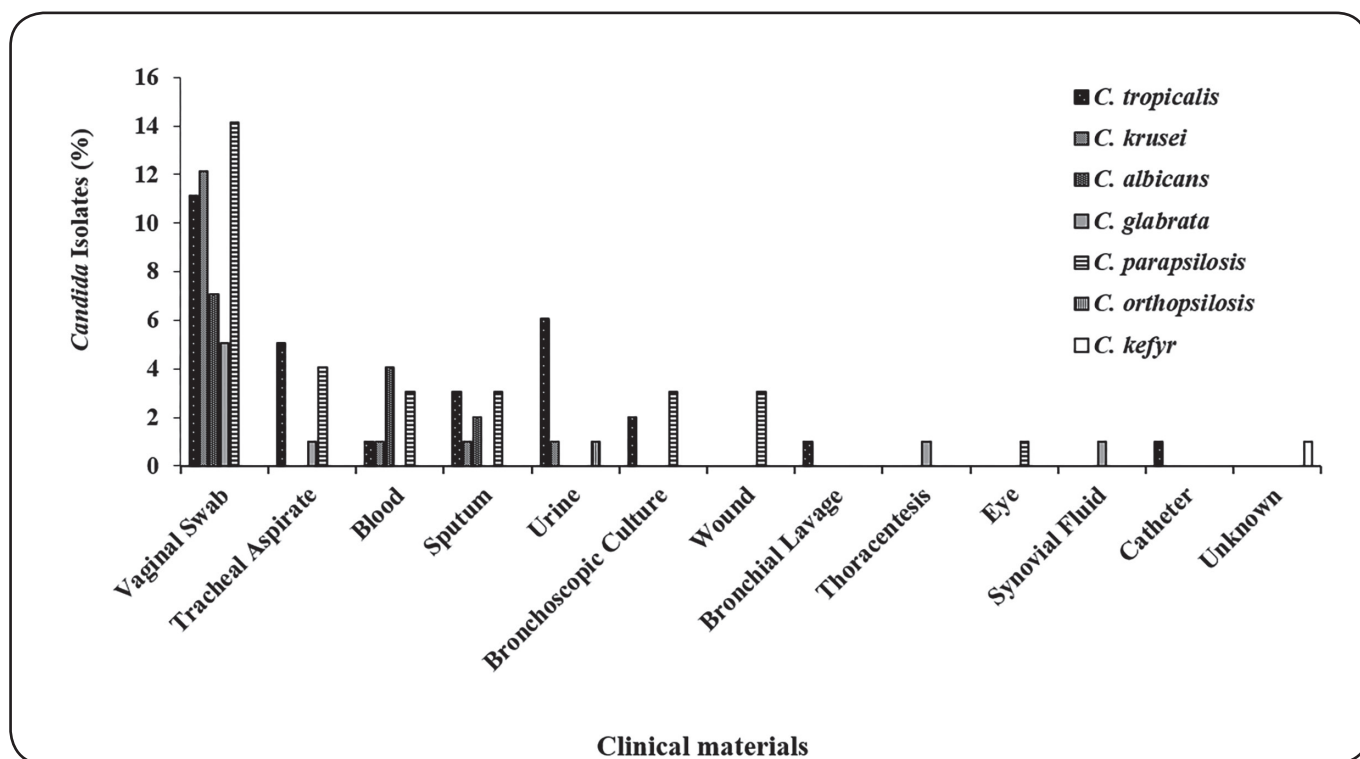


FIGURE 1: Distribution of different *Candida* species in various clinical materials (n = 99 isolates).

TABLE 2: Frequencies of non-biofilm-forming, low biofilm-forming, intermediate biofilm-forming, and high-biofilm forming *Candida* species (n = 99 isolates)\*.

	NBF (%)	LBF (%)	IBF (%)	HBF (%)
<i>C. albicans</i>	13.1	0.0	0.0	0.0
<i>C. glabrata</i>	5.1	0.0	2.0	1.0
<i>C. kefyr</i>	0.0	1.0	0.0	0.0
<i>C. krusei</i>	15.2	0.0	0.0	0.0
<i>C. orthopsilosis</i>	0.0	0.0	0.0	1.0
<i>C. parapsilosis</i>	25.3	3.0	0.0	3.0
<i>C. tropicalis</i>	6.1	0.0	1.0	23.2

**NBF:** non-biofilm-forming; **LBF:** low biofilm-forming; **IBF:** intermediate biofilm-forming; **HBF:** high biofilm-forming; **OD:** optical density. \*Biofilm groups have been generated according to biofilm formation OD of tested *Candida* isolates given as follows: NBF:  $0 \leq OD < 0.4$ ; LBF:  $0.4 \leq OD < 0.8$ ; IBF:  $0.8 \leq OD < 1.2$ ; HBF:  $OD \geq 1.2$ .

*in vitro* activity against all HBF *Candida* isolates (n = 25) tested (Bonferroni *post-hoc* test, p-value < 0.00).

## DISCUSSION

In many studies, *C. albicans* has been regarded as the most prevalent *Candida* species<sup>18,19</sup>. However, the results of the present study show that *C. parapsilosis* and *C. tropicalis* were observed in high frequencies. In addition, the frequency of *C. krusei* was higher than that of *C. albicans*, confirming that the occurrence of NCAC species such as *C. tropicalis*,

*C. parapsilosis*, and *C. krusei* is increasing<sup>11,18,20</sup> as observed in a recent study. *C. tropicalis* was indicated as the most prevalent *Candida* species among all *Candida* species isolated and is regarded as an important emerging fungal pathogen associated with high mortality rate<sup>11</sup>. In line with the results of the present study, NCAC species are shown to be more prevalent than *C. albicans* in pediatric (< 3 year) and older (> 60 year) patients than in patients from other age groups (4-18, 19-60 years) and intensive care unit (ICU) patients<sup>21</sup>. According to other studies carried out with neonates, the prevalence of *C. parapsilosis* was



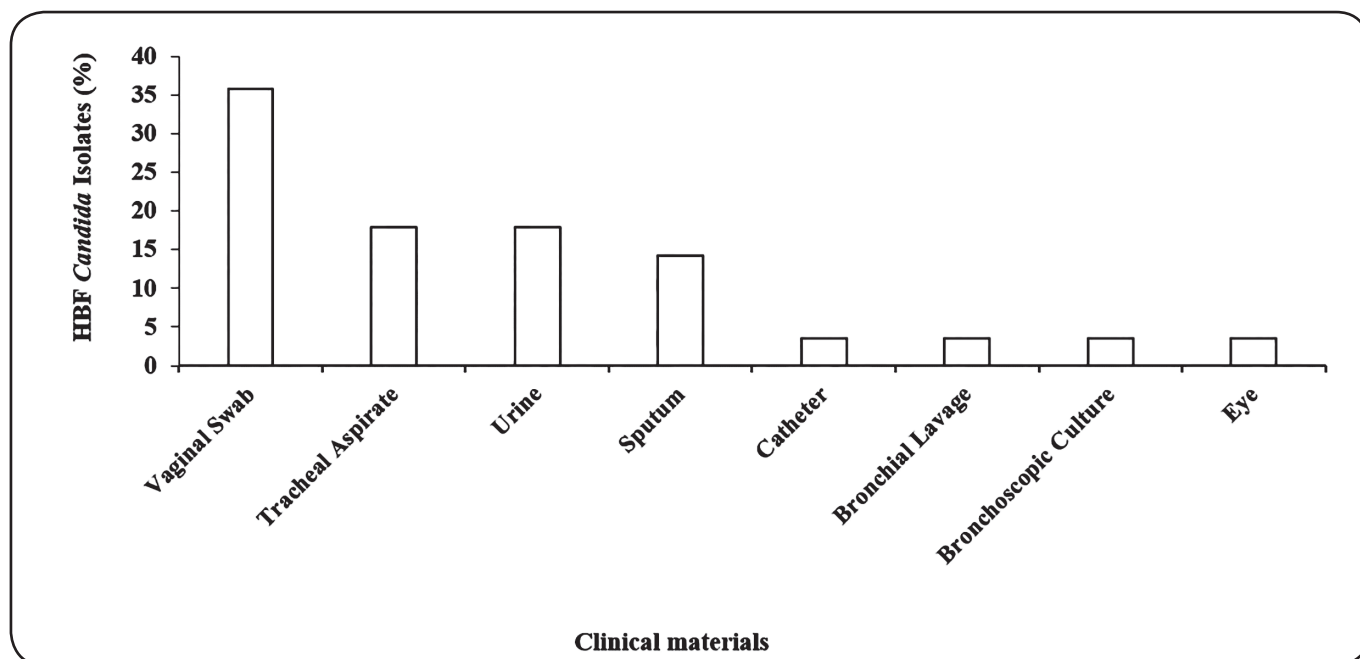


FIGURE 2: Distribution of HBF *Candida* isolates in different clinical materials ( $n = 28$  isolates). HBF: high biofilm-forming.

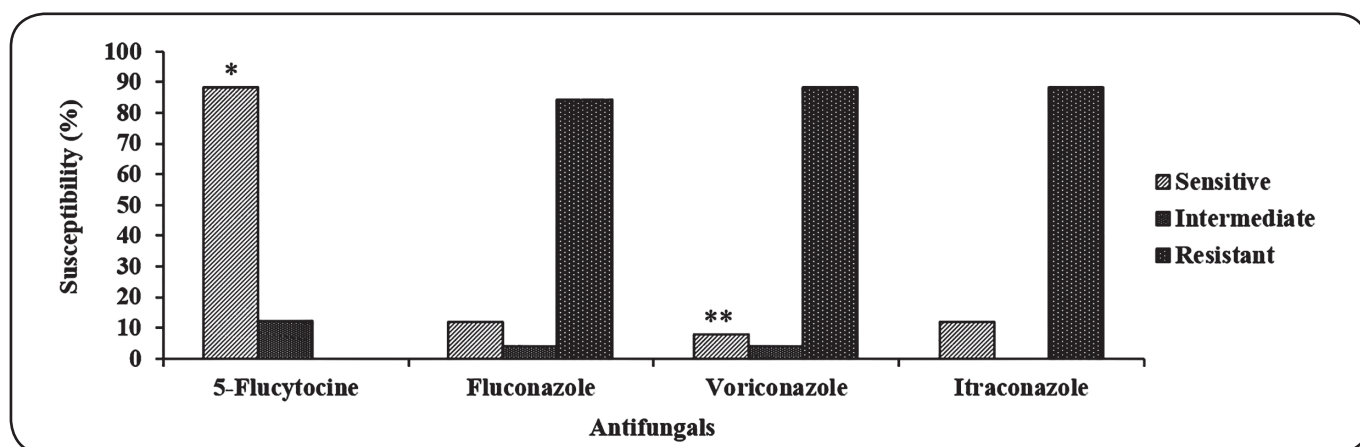


FIGURE 3: Antifungal susceptibilities of HBF *Candida* isolates (biofilm formation  $OD \geq 1.60$ ) ( $n = 25$  isolates.). All antifungal agents had significantly different effects (Chi-square test,  $p$ -value  $< 0.05$ ). HBF: high biofilm-forming; OD: optical density. \*Indicates that the tested HBF *Candida* isolates were significantly more sensitive to 5-flucytosine than to other antifungal agents used in this study (Bonferroni *post-hoc* test,  $p$ -value  $< 0.00$ ). \*\*Indicates that the tested HBF *Candida* isolates were significantly less sensitive to voriconazole than to other antifungal agents used in this study (Bonferroni *post-hoc* test,  $p$ -value  $< 0.00$ ).

higher than that of *C. albicans*, and *C. parapsilosis* has been indicated as a predominant pathogen of invasive candidiasis in neonates<sup>22,23</sup>.

Urine, vaginal swab, blood, indwelling biomaterial, and respiratory tract samples are found to be the most prevalent specimens for *Candida* isolation<sup>11,21,24</sup>. In parallel with these findings, the majority of *Candida* isolates were isolated from vaginal swab specimen, followed by specimens of tracheal aspirate, blood, sputum, and urine. However, *C. tropicalis* was found as the most frequent *Candida* species in tracheal aspirate and sputum specimens (Figure 1), contradicting the results of previous studies on the predominance of *C. albicans* in lower respiratory tract specimens<sup>12,25</sup>. The most prevalent *Candida* species

isolated from blood was *C. albicans*, confirming that *C. albicans* remains the most frequent fungal species in blood specimen<sup>18</sup>.

The investigation of the distribution of *Candida* species in different biofilm groups showed that *C. tropicalis*, which was more frequently isolated in this study, was also found as the most prevalent HBF *Candida* species, whereas all other *C. albicans* isolates were NBFs (Table 2). Therefore, the predominance of *C. tropicalis* instead of *C. albicans* was thought to be related to its enhanced biofilm formation ability<sup>24</sup>.

Applications of temporary or permanent biomaterials and medical devices in medicine have particularly led to an increase in the incidence of biofilm-associated infections<sup>26,27</sup>. One of the clinical specimens positive for HBF *Candida* isolates was

catheter (**Figure 2**). Furthermore, HBF *Candida* isolates were more frequently isolated from the clinical specimens (vaginal swab, tracheal aspirate, and urine) that were related to the body parts that may be exposed to biomaterials such as intrauterine devices, endotracheal tubes, and urinary catheters.

The treatment of invasive fungal infections is usually carried out with five major groups of antifungal agents, including azoles, polyenes, allylamines, echinocandins, and pyrimidine analogues<sup>28</sup>. Fluconazole, voriconazole, and itraconazole belong to azole class, while 5-flucytosine is a pyrimidine analogue (**Figure 3**). Of these, azoles that target ergosterol biosynthesis via blockage of the enzyme lanosterol 14 $\alpha$ -demethylase<sup>28</sup> are the most widely used group of antifungal agents<sup>29</sup>. A recent study evaluating the susceptibilities of different *Candida* species to fluconazole, voriconazole, itraconazole, ketoconazole, and 5-flucytosine showed that the majority of *Candida* isolates were sensitive to fluconazole and 5-flucytosine<sup>30</sup>. However, 5-flucytosine known to inhibit both ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) synthesis after being converted to 5-fluorouracil<sup>31</sup> was the most effective antifungal agent against all HBF *Candida* species tested in the present study (**Figure 3**).

The high resistance to fluconazole may be mainly related to the high biofilm formation ability of the tested *Candida* isolates. Biofilms are known as suitable environments for horizontal gene transfer mechanisms<sup>32</sup>. Therefore, high biofilm formation ability may play an important role in the acquisition of new antifungal resistance traits in various *Candida* species.

*Candida tropicalis* isolates that demonstrated high biofilm formation capacity were shown to display higher rate of resistance to fluconazole in one of the recent studies<sup>33</sup>.

In conclusion, the present study shows that *C. tropicalis* displays the highest biofilm formation ability among the *Candida* species evaluated. Our findings indicate high frequency of HBF *Candida* isolation from clinical samples of vaginal swab, tracheal aspirate, and urine. We also found that 5-flucytosine is the most efficient antifungal agent against HBF *Candida* isolates.

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#### Conflict of interest

The authors declare that there is no conflict of interest.

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