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Research Article

Typing of Candida Strains Straightened from Immunesupressed Patients and Investigation of Their Sensitivity to Antifungal Drugs by E-Test Method

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Abstract

Objective: Investigation of the Antifungal Susceptibility of Candida Strains Isolated from Immune Suppressed Patients, with E test Method It is well-known that Candida causes a considerable mortality via leading invasive and disseminated-systemic infections in the patients whose immune suppressed or in poor clinic condition.

Materials and Methods: In this study, it was aimed to investigate typing of Candida species that were isolated from various clinic samples from inpatients whose immune-suppressed or in poor clinic condition and also to determine the antifungal susceptibility of these isolates. During the study, a total 46 Candida were isolated from urine (6), sputum (6), blood (11), wound (4), endotracheal aspirate (7), nasopharynx (3), catheter (7) and stool (2) samples of 39 febrile neutropenic patients and 7 patients with poor clinic condition in intensive care units. Typing of the Candida isolates were carried out with API ID 32 C (bio Mérieux) kits. Antifungal susceptibility was determined with the E test (AB Biodisk, Sweden).

Result: Of the strains included into the study, 24 (52.1%) were identified as Candida albicans, five (10.8%) were Candida krusei, seven (15.2 %) were Candida tropicalis, four (8.6 %) were Candida glabrata, three (6.5 %) were Candida parapsilosis and three (6.5%) were Candida guillermondii. In the susceptibility assay with E test, 2.1% of the isolates were found resistant to amphotericin B, 21.7 % to ketoconazole and fluconazole, 17.3 % to voriconazole, 23.9% to itraconazole and 6.5 % to flucytosine. The MIC50 and MIC90 values of the most active antifungal, amphotericin B, were determined as 0.019 and 0.75 µg/ml, respectively; and the MIC50 and MIC90 values of the least active antifungal, itraconazole, were determined as 0.032 and 16 µg/ml, orderly.

Conclusion: Due to development of the medicine, the target patient population of Candida, which is an opportunistic pathogen, is progressively augmented, and by the time, it is observed that the prevalence of the invasive candida infections are being increased. Therefore, to successfully treat the serious infections caused by Candida in the patients at high risk, typing of the Candida should be routinely performed and antifungal susceptibility of these strains should be investigated with a reliable method, regularly.

Keywords: Candida strains; Amphotericin B; Voriconazole; Ketoconazole; Itraconazole; Fluconazole; Flucytosine; Antifungal susceptibility

Introduction

The increase in fungal infections in recent years is directly proportional to the widespread use of broad-spectrum antibiotics and immunosuppressive drugs, increasing invasive procedures and the widespread use of major surgical interventions [1]. Candida species often cause opportunistic infections in patients with poor general condition or immunocompromised. Candida species, which are

responsible for 86% of fungal infections in such patients, are the fourth most frequently isolated microorganisms from blood cultures [2]. Candida species are also responsible for 8-12% of nosocomial sepsis [3].

candidemia caused by candida; It is a serious clinical picture with a high mortality rate and difficult to diagnose and treat [4,5]. In mycological diagnosis, molecular methods are applied together with conventional diagnostic methods. [5-9] It causes prolonged hospital stay and has a higher mortality compared to sepsis developing with bacterial pathogens [4].

Although there are more than 150 candida species in nature, only some of them have been determined to cause disease in humans. These species are; *Candida albicans* (*C. albicans*), *Candida tropicalis* (*C. tropicalis*), *Candida krusei* (*C. krusei*), *Candida glabrata* (*C. glabrata*), *Candida guiiliermondii* (*C. guiiliermondii*), *Candida stellotoidea* (*C.stellotoidea*), Candida kefir (C.kefyr), *Candida lusitania* (*C. lusitania*) and *Candida dubliniensis* (*C. dubliniensis*) [5]. Although *C. albicans* is the most frequently isolated species, the number of infections caused by non-albicans species is increasing [3,4,10].

Antifungal drugs are effective against local or systemic infections of the skin, mucous membranes and organs; are agents that can be administered topically, orally or parenterally. Antifungal drug therapy was limited to potassium iodide and methylene blue until the 1950s due to its toxic effects. Later, amphotericin B was discovered and it has maintained its importance in the treatment of systemic fungal infections since its introduction in the 1950s [11]. Systemic-acting antifungal drugs are few in number. The discovery of azole derivatives such as miconazole, ketoconazole, itraconazole, and fluconazole after amphotericin B led to significant improvements in treatment [12].

5-flucytosine, which is an alternative to amphotericin B, came into use in 1964. In the late 1960s, imidazole group antifungal drugs, which formed the first group of synthetically obtained azoles; It consists of clotrimazole, miconazole, econazole, isoconazole, ketoconazole and fenticonazole. After 1980, terconazole, itraconazole and fluconazole from triazoles, which are the second azole derivatives, came into clinical use. Itraconazole and fluconazole, which are called second generation azoles, have become alternatives to amphotericin B in the treatment of invasive infections caused by many different fungal species, due to their clinical effectiveness and safety. In particular, the success of fluconazole has been encouraging for the development of new third generation azoles (second generation triazoles). Voriconazole, pseoconazole and ravuconazole are second generation triazoles, and although they have been used in our country in recent years, there are few studies on their clinical effectiveness [1,11-14].

While resistance to the antifungal drugs used today was very rare until 10 years ago, in the 1990s, resistance, especially in immunocompromised patients, started to become an important problem. For example, it has been reported that resistance to antifungal drugs often occurs in oropharyngeal candida infections in patients with AIDS [15].

In this study, we aimed to differentiate the species of candida isolated from various clinical samples taken from patients with poor general condition or immunocompromised patients, who are considered as high-risk patients for candida infections, and to determine these strains by using the E test (AB Biodisk, Sweden) method with amphotericin B, flucytosine, fluconazole. It was aimed to determine the susceptibility of antifungal drugs such as ketoconazole, voriconazole and itraconazole.

Materials and Methods

In this study, 46 candida strains isolated from various clinical samples sent to the Microbiology Laboratory from patients who were hospitalized in different clinics of Fırat University Fırat Medical Center between January 1st and August 31st, 2005 and whose general condition was impaired due to various diseases or whose neutrophil count fell below the critical level; It was examined in terms of species identification and detection of antifungal susceptibility. Some epidemiological information of the patients, such as age, gender, major disease, hospital admission, risk factors for fungal infection, were recorded. Clinical samples sent to our laboratory for microbiological examination were cultivated in blood culture bottles (Organon or Becton Dickenson blood culture bottles), blood agar, EMB and/or Sabouraud Dextrose Agar medium (SDA) according to the type of sample. Gram staining was done first from cultures that produced pure yeast colonies. Samples with Gram (+) yeast cells in Gram staining were passaged into SDA medium and incubated at 30° C for 24-48 hours. SDA growing yeasts; Colony structures, typical yeast cells appearance on Gram stain, germ tube formation, presence of true or false hyphae in Tween 80 Corn Meal Agar, chlamidospore structures were examined. API ID 32 C (Bio-Merieux, France) strips were used for the biochemical evaluation of yeasts.

Colonies of yeasts taken from fresh cultures (24 hours) in SDA medium with the help of sterile wipes were mixed in 0.85% suspension medium. This suspension was spread on RPMI 1640 medium prepared in 90 mm petri dishes with the help of cotton-tipped non-adsorbent sterile wipes. Two E-test antifungal strips were placed in the middle of the medium. The plates were covered with paraffin to retain moisture. The prepared plates were incubated for 24-48 hours at 350° C. When the inhibition ellipses formed at the end of the incubation were evaluated, the value at which growth was completely inhibited (100% inhibition) for amphotericin B and flucytosine in accordance with the manufacturer's and NCCLS recommendations; For azoles, the value at which growth was inhibited by 80% was accepted as the MIC value for that drug. (11th). According to the obtained MIC values, the susceptibility or resistance status of the strains to the tested antifungal was interpreted in accordance with NCCLS M27A recommendations. The strains were classified as susceptible, less susceptible or resistant according to their MIC values [11].

Results

Distribution of Candida Species 39 of the 46 candida strains included in the study were isolated from febrile neutropenic patients. The remaining 7 strains were isolated from comatose patients in

the surgical intensive care unit. of the studied candida, 24 (52.1%) *C. albicans*, five (10.8%) *C. krusei*, seven (15.2%) *C. tropicalis*, four (8.6%) *C. glabrata*, three (6.5%) *C. parapsilosis* and three (6.5%) were identified as *C. guillermondii*. The distribution of Candida species according to the clinical samples from which they were isolated is shown in Table 1.

Table 1: Distribution of Candida Species According to the Clinical Samples from which They wereIsolated.

Clinical Example	*C.albicans*	*C.krusei*	*C.tropicalis*	*C.glabrata*	*C.parapsilosis*	*C.guillermondii*
Urine (n:6)	2	-	1	-	3	-
Sputum (n:6)	2	1	1	2	-	-
Blood (n:11)	7	1	2	1	-	-
YarWound (n:4)	-	1	1	-	-	2
ETA (n:7)	6	1	-	-	-	-
Throat (n:3)	1	-	1	1	-	-
Catheter (n:7)	5	1	1	-	-	-
Stool (n:2)	1	-	-	-	-	1
Total (N:46)	24	5	7	4	3	3

ETA: Endotracheal aspirate; C: Candida

The MIC ranges of the drugs tested against candida species in the antifungal 35 susceptibility experiment performed with the E test method for the candida strains included in the study are shown in Table 2.

Table 2: MIC Ranges of Antifungal Drugs Detected by Candida Species.

Candida Species	MIC Ranges (µg/ml)					
	Amphotercin B	Ketoconazol e	Voriconazol e	Itraconazol e	Fluconazol e	Flucytosin e
C. albicans	0.008-0.75	0.004-16	0.006-16	0.006-16	0.094-196	0.064-4
C. krusei	0.0023-1	0.125-≥32	0.125-32	0.38-≥32	0.5-256	0.50-32
C. tropicalis	0.016-0.75	0.023-16	0.38-12	0.023-16	1-16	0.125-≥32
C. glabrata	0.016-4	0.125-16	0.016-8	0.125-≥32	0.5-≥256	0.38-≥32
C. parapisi	0.023-0.50	0.006-0.50	0.032-2	0.006-1	0.125-12	0.75-1
C. guillermondi	0.019-1	0.006-2	0.19-4	0.006-1	0.006-8	0.016-0.50

C: Candida

Resistance to amphotericin B was detected in only one (2.1%) *C. glabrata* strain out of a total of 46 candida strains (4 µg/ml). The MIC distribution of amphotericin B against the studied candida strains is shown in Figure 1.

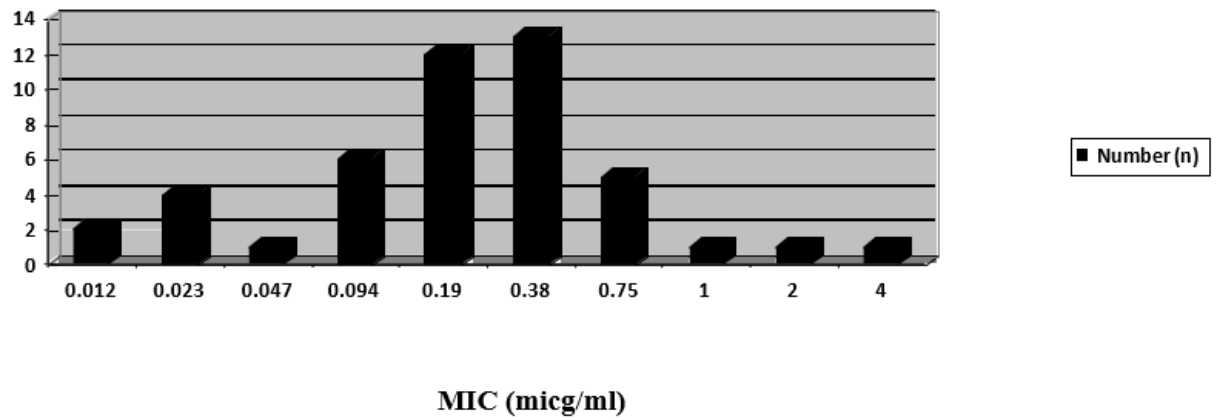

Figure 1: Distribution of MIC Values Measured Against Amphotericin B in Candida Strains.

In the study, resistance to flucytosine was found in a total of three (6.5%) candida species, including one *C. krusei*, one *C.tropicalis*, and one *C. glabrata* strain. The MIC distributions of flucytosine against the strains tested are shown in Figure 2. No strains less susceptible to flucytosine were detected.

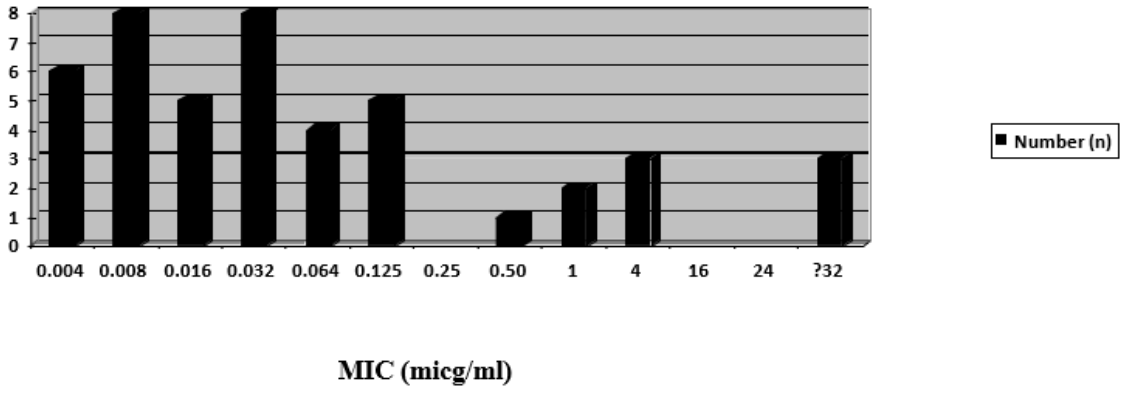

Figure 2: Distribution of MIC Values Measured Against Flucytosine in Candida Strains.

In the study, fluconazole resistance was observed in a total of 10 (21.7%) candida species, including two *C. albicans*, four *C. krusei* and four *C. glabrata*. Less sensitivity was detected in two *C. parapsilosis* and one *C. tropicalis* strains (16-32 µg/ml) (Figure 3).

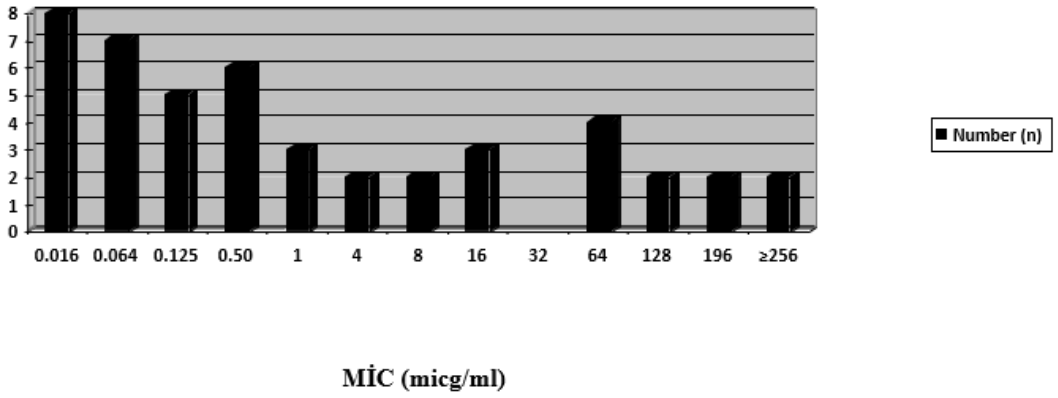

Figure 3: Distribution of MIC Values Measured Against Fluconazole in Candida Strains.

In the study, itraconazole resistance was observed in 11 (23.9%) candida species, three of which were C. *albicans*, four *C. krusei*, four C. glabrata and one C. tropicalis. Less susceptibility was detected in one *C. parapsilosis* and one *C. guillermondii* strain (1 µg/ml) (Figures 4&5).

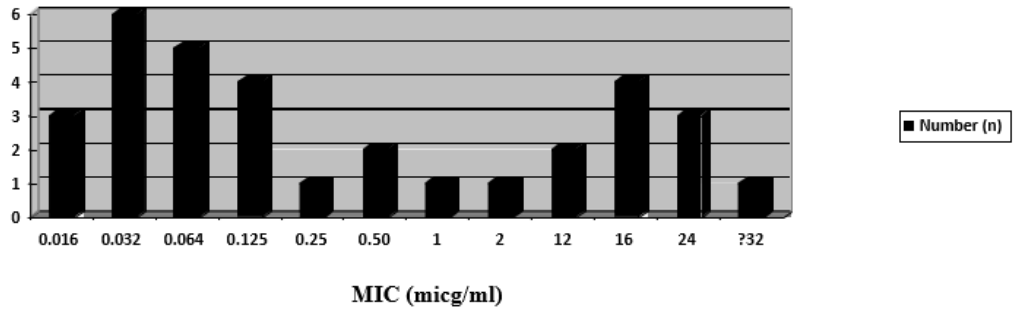

Figure 4: Distribution of MIC Values Measured Against Ketoconazole in Candida Strains.

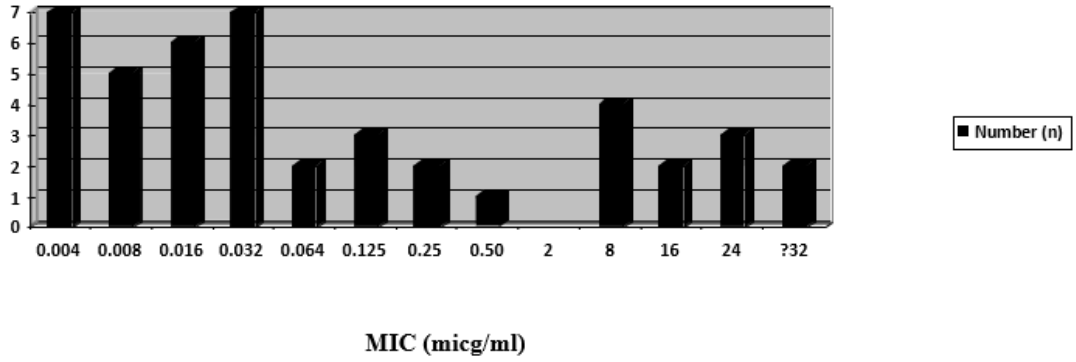

Figure 5: MIC Values Measured Against Itraconazole in Candida Strains.

In the study, voriconazole resistance was observed in a total of eight (17.3%) candida species, including two *C. albicans*, three *C. krusei*, and three *C. glabrata*. Less sensitivity was detected in one *C. guillermondii* and one *C. parapsilosis* strain (2 and 4 µg/ml) (Figure 6).

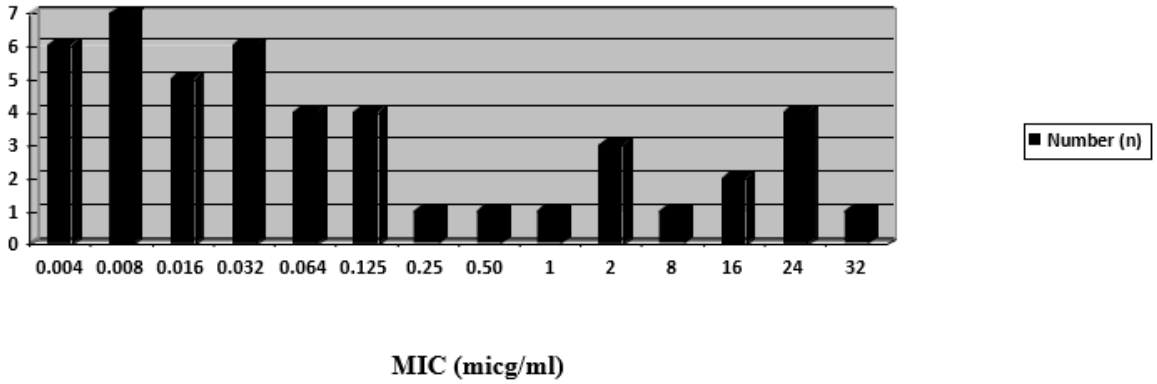

Figure 6: MIC Values Measured Against Voriconazole in Candida Strains.

The MIC50, MIC90 values of the antifungal drugs tested against the Candida species included in the study and the antifungal resistance rates according to the species are calculated and shown in Table 3.

Table 3: MIC50 and MIC90 Values of Antifungal Drugs and Resistance Rates by Strains.

Candida Turleri (N)	Antifungal Resistance Frequency%					
	AB	KT	VR	IT	FL	FC
C. albicans (24)	0	8.3	8.3	12.5	8.3	0
C. krusei (5)	0	60	60	80	80	20
C. tropicalis (7)	14.2	14.2	0	14.2	0	14.2
C. glabrata (4)	0	75	75	100	100	25
C. parapisilosis (3)	0	0	33.3*	33.3*	66.6*	0
C. guillermondii (3)	0	33.3*	33.3*	33.3*	33.3*	0
MIC50	0.019	0.064	0.032	0.032	0.5	0.032
MIC90	0.75	16	16	16	128	4

C: Candida; AB: Amphotericin B: AB; KT: Ketoconazole; VR: Voriconazole; IT: Itraconazole; FL: Fluconazole; FC: Flucytosine

Discussion

Antifungal drug therapy was limited to methylene blue and potassium iodide until amphotericin B came into clinical use [6]. Flucytosine after amphotericin B; and then azoles were started to be used [12]. Since the 1990s, resistance has started to become an important problem especially in immunosuppressed patients [15]. Candidas are yeasts that live as hosts in many body flora such as the gastrointestinal and uro-genital system, skin, and upper respiratory tract, and there are more than 200 taxonomic species [17]. Only 10% of candida are pathogenic for humans [17]. Five species have been identified in more than 95% of the Candida infections detected in humans. These agents are *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. krusei* and *C. glabrata* [18,19].

In healthy people, they mostly cause self-limiting, easy to diagnose, superficial and mucosal infections. Such infections can generally be cured with simple hygienic measures and local drug treatments [20,21]. Although life-threatening invasive and systemic infections due to candida can be observed rarely, most of them develop in individuals whose immune system is suppressed or whose general condition is severely impaired [22]. Invasive candidiasis is a common term for two different clinical conditions. One of them is candidemia, and the other is systemic or diffuse candidiasis. Candidemia is the isolation of a candida species from blood accompanied by positive infective findings. Sometimes clinical findings may not be detected in neutropenic patients, graft recipients or patients receiving steroids. Systemic or diffuse candidiasis is the culture or histopathological detection of the presence of a candida species in a tissue (in sterile areas) that should not be. However, it is generally used for invasive candidiasis, mostly hematogenous candida infections. On the other hand, although it has been reported that invasive candidiasis is seen approximately 2 times more in cancer or transplantation centers compared to other hospitals, common candida infections in hospitals providing tertiary health care with intensive care units are increasing their importance. In the light of this information, it is understood that invasive infections due to candida are no longer rare clinical conditions and are not limited to neutropenic and immunosuppressed patients. Intensive care patients with poor general condition and dependent on life support units have been an important target population for serious systemic candida infections [23]. Mortality in candidemia varies between 20-30%. Opportunistic mycoses also cause prolonged hospitalization and increased cost [24,25].

Most of the candida infections in humans are caused by *C. albicans*. This is thought to be due to the fact that *C. albicans* is the most common species in the flora [1,2,4,23]. In our study, the most frequently isolated species from clinical specimens was *C. albicans* (52%). This was followed by *C. tropicalis* (15%) with the second frequency, and the least defined species were *C. parapsilosis* and *C. guillermondii* (7%). Gultekin B, et al. [26], In a study they conducted and used different identification methods, 56% of 94 Candida species were *C. albicans*, 20% *C. glabrata*, 10% *C. tropicalis*, 5% *C. kefyr* and 1% *C. dubliniensis*. They defined it as *C. dupliniensis* [26]. In a study reported by Ozcelik et al. [27], in 2004, using ID 32 C kits, *C. albicans* was the most frequently identified species (77%) and the least frequently isolated species was C. sake (0.9%) out of a total of 104 clinical isolates [27]. Tumturk A, et al. [28], of the candida isolated from the blood cultures of the patients hospitalized in the intensive care unit, 75 (49.1%) of the candida were *C. albicans* and 78 (50.9%) were non-albicans candida (NAC). NACs; 32 (21%) *C. parapsilosis*, 21 *C. glabrata* (13.7%), 7 (4.6%) *C. tropicalis*, 6 (3.9%) *C. lusitaniae*, 5 (3.2%) consisted of *C. lipolytica* and 7 (4.6%) other candida growths [28]. Togay A, et al. [29], AuxaColor evaluated 71 candida strains isolated from various clinical specimens of patients hospitalized in the intensive care unit, and 28 (39%) *C. albicans*, 13 (18.3%) *C. parapsilosis*, 11 (15.5%) *C. glabrata*, Ten (14.1%) were identified as *C. tropicalis*, four (5.6%) as *C. krusei*, three (4.2%) as *C. lusitaniae*, and one (1.4%) as *C. kefyr* and *C. dubliniensis* [29]. Beder D, et al. [30], in their study, they identified *C. albicans* with 41.3% and *C. parapsilosis* with 38% [30]. Altin N, et al. [31], of 51 Candida strains isolated from various clinical specimens of patients hospitalized in the intensive care unit, 26 (51%) *C. albicans*, 7 (14%) *C. tropicalis*, 5 (10%) *C. parapsilosis*, 4 (8%)) *C. glabrata*, 3 (6%) *C. krusei*, 3 (6%) *C. lusitaniae*, 2 (4%) *C. famata* and 1 (2%) *C. kefyr* [31]. Muderris T, et al. [32], in the study in which they investigated deaths due to candidemia, *C. parapsilosis* was the most common 49.1%, followed by *C. albicans* 26.9% and *C. tropicalis* 7.9% [32]. Species distribution of candida varies according to the center of study, the patient population studied and the characteristics of the geographical regions [23].

In our study, when the clinical samples from which candida were isolated, 11 of 46 strains were obtained from blood, 7 from endotracheal aspirate and catheter, 6 from sputum and urine, 4 from wound, 3 from throat and 2 from feces. *C. albicans* was the most isolated candida species from almost all clinical specimens. Among the antifungals tested in our study, the most effective drug was found to be amphotericin B. Moderate (4 µg/ml) amphotericin B resistance was observed in only one *C. glabrata* strain (2%) out of a total of 46 strains studied. Both MIC50 and MIC90 values for amphotericin B were within the sensitive limits, respectively, as 0.019 µg/ml and 0.75 µg/ml. The second most effective drug among the antifungals whose efficacy was measured in our study was found to be flucytosine (93.5%). Among all strains, one *C. krusei*, one *C. tropicalis* and one *C. glabarata* strain were found to be resistant to flucytosine at MICs of 32 µg/ml, ≥32 µg/ml and ≥32 µg/ml, respectively. Both MIC50 and MIC90 values for flucytosine were within the sensitive limits, measuring 0.032 µg/ml and 4 µg/ml, respectively. In our study, the most effective antifungal from the azole group was voriconazole (82.7%); the least sensitive antifungal was found to be itraconazole (76.1%). Ketoconazole and fluconazole were observed as moderately active antifungals among azoles and their sensitivity was found to be 78.3%.

In the study conducted by Ozcelik B, et al. [27], 100% of 104 candida strains were found to be sensitive to amphotericin B and voriconazole, and 93% to flucytosine and fluconazole [27]. In another study by Kaya S, et al. [33], the antifungal susceptibility of 97 candida, of which 70% of the strains were hospital-acquired, was measured with a commercial kit (Candifast/Italy) and the sensitivity of these strains to amphotericin B 47 100%, flucytosine sensi-

tivity 98.9%, ketoconazole sensitivity was %. 90 and fluconazole sensitivity has been reported to be 89% [33]. Yilmaz B, et al. [34], the efficacy of amphotericin B, fluconazole, and itraconazole in 100 candida strains was measured by the E test method, and their sensitivities were 98%, 58%, and 56%, respectively, in C. albicans strains; efficacy in non-albican strains was 96%, 44% and 64%, respectively. The measured MIC50 and MIC90 values for amphotericin B, fluconazole and itraconazole were 0.125-0.75, respectively; It was determined as 0.50->256 and 0.125->32 µg/ml [34].

A *C. parapsilosis* strain moderately susceptible to Amphotericin B and a resistant *C. tropicalis* of 47 strains evaluated by Fungitest were examined by the Etest method, and the minimum inhibitory concentration (MIC) was 1.5 µg/ml. Three *C. albicans* strains moderately susceptible to fluconazole (FLU) were evaluated with the E test method, and MIC values of two were found as 256 µg/ml and one as 32 µg/ml. The MIC value of one t strain resistant to FLU was found to be 256 µg/ml by the E test method. The MIC value of the resistant C. tropicalis strain was found to be 0.38 µg/ml [24]. Altın N et al. [31], while flucytosine was susceptible in 47 strains (95.9%) in candida isolated from intensive care patients, moderate in 2 strains of *C. krusei*, fluconazole was susceptible in 42 strains (85.7%), and resistant in 6 strains (3 of them *C. krusei*), 3 *C.glabrata*), 1 *C. glabrata* strain was moderately susceptible, while voriconazole was sensitive in 48 (98%) strains, 1 *C. glabrata* strain was resistant. While amphotericin B was sensitive in 44 (90%) strains, 4 strains (2 *C. krusei*, 2 *C. glabrata*) were found to be moderately susceptible [31].

In our study, the antifungal susceptibility status of candida, mostly isolated from immunocompromised patients with invasive candidiasis, was investigated using the E test method; amphotericin B and flucytosine were found to be the most effective drugs. The efficacy of the azole antifungals included in the study was found to be quite low, especially against non-albicans species. Therefore, when starting empirical treatment in candida infections, at least making the species distinction of the pathogen will be a factor that can increase the success of the treatment. With the rapid spread of AIDS, the development of organ or tissue transplantation, more frequent invasive interventions and major surgeries, the increase in the number of elderly and debilitated patients with the prolongation of life expectancy, the more frequent use of chemotherapeutics that are toxic to the bone marrow due to malignant diseases, and the widespread use of intensive care units, life support units are required. As a result of the increase in the number of patients with diabetes mellitus, the patient population targeted by candida is increasing.

Conclusion

The importance of candida, which is at least as a cause of mortality and morbidity as much as bacterial pathogens, has increased. For the treatment of invasive and widespread candida infections, especially in risky individuals, it will be beneficial to start routine identification of species and antifungal susceptibility tests, and to establish antifungal use protocols that will minimize the development of resistance.

Ethics Committee Approval

Approval was obtained from the Ethics Committee of the Republic of Turkey, Fırat University (Decision No: 14/6 Date: 30.12.2004).

Acknowledgement

None.

Conflict Of Interest

No conflicts of interest related to this article were declared by the authors.

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