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ORIGINAL ARTICLE

Antifungal Susceptibility Testing and Cluster Analysis of *Candida auris* Strains

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SUMMARY

Background: Candida auris **is an opportunistic pathogen that has become widespread in recent years and shows resistance to multiple drugs. The aim of our study was to determine the antifungal susceptibilities of *C. auris* isolates, to perform a dendrogram from the mass spectra of strains obtained from matrix-assisted laser desorption ionization-time of flight mass spectrometry in order to evaluate the proteomic similarities of the strains and determine the geographical clade of *C. auris* strains in this study by the help of Multiplex RT-PCR.**
Methods: **The samples yielded 58 *C. auris* isolates. MALDI TOF MS (BioMerieux, France) was used for identification of the isolates and Sensititre Yeast One (Thermoscientific) system was used for antifungal susceptibility testing. Dendrograms of strain's spectra were generated by using the RUO/Saramis (BioMerieux, France) database and evaluated through hierarchical clustering analysis. The selected nine strains were examined at the clade level by using Multiplex RT-PCR.**
Results **The susceptibility profile of the strains revealed resistance to Fluconazole in 84% (MICs $\geq$ 32) and resistance to Amphotericin B in 60% (MIC $\geq$ 2). All strains were found to be sensitive to Anidulafungin and Micafungin. The dendrogram of the main spectra of *C. auris* isolates showed a similarity range of 35 - 100%. The nine strains studied were identified as clade 1 (South Asian).**
Conclusions: **It was determined that *C. auris* strains were members of geographical clade 1, and the Amphotericin B resistance was found to be higher than expected. This situation poses a threat to critically ill patients.**
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KEYWORDS

C. auris, antifungal susceptibility, MALDI-TOF MS, dendrogram, geographical clade

LIST OF ABBREVIATIONS

MALDI-TOF MS - Matrix-assisted laser desorption ionization-time of flight mass spectrometry
RT-PCR - Reverse transcription-polymerase chain reaction

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INTRODUCTION

Candida auris is an ascomycetous yeast belonging to the *Saccharomycetes* class. Phylogenetically, it is a member of the *Clavispora* clade of the *Metschnikowiaceae* family, along with other multidrug-resistant yeast species such as *C. haemulonii*, *C. duobushaemulonii*, and *C. pseudohaemulonii* (the *C. haemulonii* species complex) [1].

C. auris reproduces in ovoid, ellipsoidal, or elongated cells ranging from 2.0 - 3.0 x 2.5 - 5.0 µm in size. Its optimum growth temperature is 37 - 40°C, although it can also grow at temperatures of 40 - 42°C [1,2]. *C. auris* is an opportunistic pathogen that has become widespread worldwide over the past decade, causing nosocomial outbreaks. Difficulties in identifying *C. auris*, its ability to survive for extended periods on contaminated patient beds and healthcare facility surfaces and its propensity for multidrug resistance, pose challenges in clinical settings [3]. While *C. auris* was first isolated globally in 2009 [4], its first report in our country was in 2021 [5], with cases reported in more than 40 countries [6]. Most infections occur as systemic infections in critically ill patients in intensive care units. While invasive infections, such as bloodstream infections, are most commonly reported, urinary tract infections, osteomyelitis, pericarditis, wound infections, skin abscesses, and cases of meningitis have also been reported [7]. The identification of colonized patients involves obtaining swab samples, particularly from the axilla and groin regions, yet *C. auris* has also been isolated from swabs taken from the nasal cavities, oropharynx, external ear canal, vagina, and rectum. Detecting *C. auris* colonization enables the implementation of infection prevention and control measures [8]. Mortality rates in *C. auris* candidemia are high, with hospital-specific mortality rates ranging from 25% to 70% [1]. Epidemiological data on how *C. auris* spreads among patients and healthcare facilities and how long it can persist colonized are limited, making it challenging to reduce transmission. *C. auris* colonization disrupts the microbiome on patients' skin in healthcare facilities. It can remain viable on plastic surfaces for up to 28 days and on wet and dry steel surfaces for up to 7 days [1]. Colonization of *C. auris* on hospital floors and medical equipment facilitates transmission within healthcare facilities. While no defined CLSI (Clinical and Laboratory Standards Institute) or EUCAST (The European Committee on Antimicrobial Susceptibility Testing) minimum inhibitory concentration (MIC) breakpoints exist for *C. auris* susceptibility, temporary MIC breakpoints have been proposed [9]. Therefore, monitoring epidemiological data related to *C. auris* is crucial. Thus far, five different geographical clades have been identified and numbered based on location for *C. auris* isolates: South Asia (I), East Asia (II), Africa (III), South America (IV), and Iran (V). Different clades exhibit more than 35,000 single nucleotide polymorphism differences in whole-genome sequence analysis (WGS). Clades differ in geographical distribution, phenotypic characteristics, antifungal susceptibility profiles, outbreak potential, and clinical symptoms [1,10]. Finally, a sixth clade has been identified in Singapore [11]. The global spread of *C. auris* is thought to be facilitated by individuals exposed to *C. auris* during healthcare delivery [12].

The objective of our study was to determine the antifungal susceptibilities of *C. auris* isolates, perform a dendrogram from the mass spectra of strains obtained using MALDI TOF MS (BioMerieux, France), evaluate the proteomic similarities, and determine the geographical clade of the examined isolates by using the Multiplex RT-PCR method.

MATERIALS AND METHODS

Methodology and sample selection

The samples sent to our ISLAB-4 regional laboratory from surrounding hospitals were cultured and evaluated according to the routine microbiology procedure. *C. auris* isolates were identified by MALDI-TOF MS (BioMerieux, France) (Figure 1a and 1b) and were included in the study. The colorimetric microdilution method (Sensititre YeastOne, TREK Diagnostic Systems) was used for antifungal susceptibility testing.

Cluster analysis

The dendrogram of strain spectra from the MALDI-TOF MS (BioMerieux, France) device was created by using the RUO/Saramis (BioMerieux, France) database software and evaluated through hierarchical clustering analysis (Figure 2).

Multiplex qPCR

Based on the proximity in the dendrogram, nine *C. auris* strains were selected, and nucleic acid extraction was performed by using Zybio EXM3000. The closest 2 strains (100%), 5 strains with intermediate proximity (60 - 70%), and 2 strains with distant proximity (30 - 40%) were included in the clade study. Specific hydrolysis probes were designed using the primers listed in the Table 1. To determine the clades of *C. auris* strains, they were optimized to work in multiplex format with 2 wells. Multiplex RT-PCR using clade-specific primer probe sets with isolated samples was performed by using Bio-rad CFX96, with the thermal cycling protocol in the Table 2.

RESULTS

In the study, 58 *C. auris* strains were isolated. Most of the isolates were obtained from blood samples (38, 65%), followed by urine (13, 22%), tracheal aspirate (4, 7%), groin (2, 3%), and wound (1, 2%) specimens. The distribution of samples according to clinics is as follows: 47 (81%) samples from intensive care units (including 9 from COVID-19 ICU), 9 (16%) from pallia-

Table 1. Primer and probe sequences used for clade determination in *C. auris* strains.

Well information	Clade	Primer sequence	Primer name
1. Well	Clade 1	TTATTTGGTCTTCAATCATTGATTCCTTGC	C. auris_CSS-1F
		TACGTGTAGTGAGTAGGAATTGAGG	C. auris_CSS-1R
		ACATGCGAGAGGCCCTGGGT	C. auris_CSS-1P_FAM
	Clade 2	AGCTACACAAAATGGTTTTTTCAGAT	C. auris_CSS-2F
		CACATCATATGCCAAAGTAGTAGAGT	C. auris_CSS-2R
		GCAACCTGTCGCGGATGCCT	C. auris_CSS-2P_ROX
2. Well	Clade 3	CGATGAGAAACCCCCATCCAA	C. auris_CSS-3F
		TTTTCATTTCTATCAGTCAATACAATACGACC	C. auris_CSS-3R
		CGCAGCCATCTCGCAGCCAT	C. auris_CSS-3P_FAM
	Clade 4	GGGGGTTTTACTATATAAATTTGTATAGCTT	C. auris_CSS4F
		CTATGTAGGTCGGGATTTTCATCC	C. auris_CSS4R
		CGGCAACACTGAAATCCTAACGCCT	C. auris_CSS-4P_ROX

Table 2. Thermal cycle of the qPCR program.

qPCR program			
Total reaction volume		20µL	
Steps	Number of cycles	Temperature	Duration
Hold	1	95°C	3 minutes
Denaturation	40	95°C	15 seconds
Annealing/Extension		60°C	30 seconds
Reading			FAM/ROX

Table 3. Antifungal susceptibility ranges, MIC50, and MIC90 distributions.

Antifungal	Range	MIC50	MIC90
FLU	0.12 - 256	64	256
ITC	0.015 - 16	0.12	16
VOR	0.006 - 8	0.5	8
PZ	0.008 - 8	0.06	8
FC	0.06 - 64	0.12	1
CAS	0.008 - 8	0.25	8
MF	0.06 - 0.5	0.12	0.25
AFG	0.06 - 2	0.12	0.25
AMP	0.064 - 2	2	2

FLU - Fluconazole, ITC - Itraconazole, VOR - Voriconazole, PZ - Posaconazole, FC - 5-Flucytosine, CAS - Caspofungin, MF - Micafungin, AFG - Anidulafungin, AMP - Amphotericin-B.

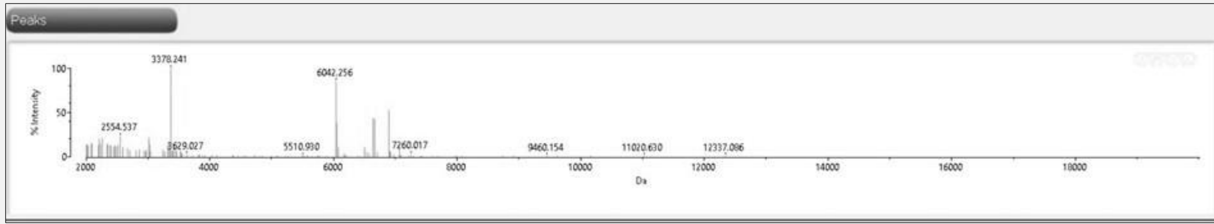

Figure 1a. ***C. auris*** **isolate spectrum obtained with MALDI TOF MS.**

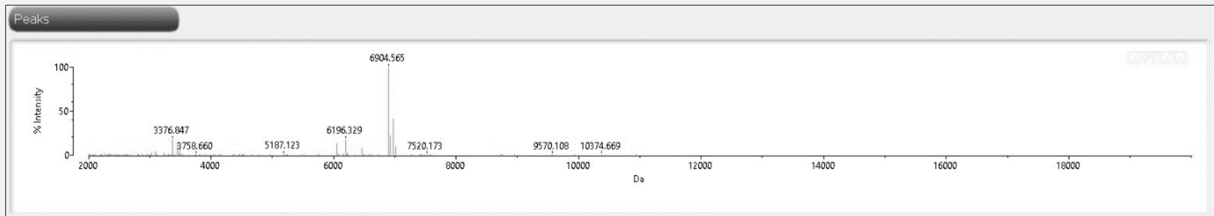

Figure 1b. Control ***C. albicans*** **isolate spectrum obtained with MALDI TOF MS.**

tive care service, and 2 (3%) from general ward inpatients.

Antifungal susceptibility data of *C. auris* isolates were evaluated, and the antifungal susceptibility ranges, MIC50, and MIC90 distributions are provided in Table 3.

According to CDC tentative breakpoint values, our study showed that (n = 49) 84,4% of the tested *C. auris* strains exhibited Fluconazole resistance (MICs ≥ 32) and (n = 35) 60,3% exhibited Amphotericin B resistance (MIC ≥ 2). All strains were found to be susceptible to Anidulafungin and Micafungin (n = 17) (29% exhibited Caspofungin resistance (MIC ≥ 2)).

The dendrogram showing the cluster analysis of 58 *C. auris* and one *C. albicans* strains using MALDI-TOF MS Ruo/SARAMIS database is shown in Figure 2. Similarity rates between isolates ranged from 35% to 100%. The closest 2 strains (100%), 5 strains with intermediate proximity (60 - 70%), and 2 strains with distant proximity (30 - 40%) were included in the clade study. The nine strains analyzed were of South Asian origin and determined as Clade 1. Amplification curves for the clades can be seen in Figure 3.

DISCUSSION

C. auris was first isolated in 2009 in Japan as a pathogen sensitive to antifungal drugs causing localized infections. Over the next decade, it evolved into different clades showing resistance to antifungal drugs at various levels in different regions of the world simultaneously [13].

In our country, the first report was made in 2021 from a university hospital in Istanbul [5], and in the same year, a strain was isolated from a blood culture sample of a patient in our laboratory. Since the identification of *C. auris* using conventional and biochemical methods is difficult, data on its incidence and prevalence are not yet clearly established, and epidemiological data are limited. It has been observed that misidentification also occurs with many automated systems [14]. With advancing technology, detecting rare strains has become easier. MALDI-TOF, a rapid and reliable method for identifying *C. auris* from pure culture, can identify *C. auris* strains at the species level with 100% sensitivity and specificity, using updated commercial MALDI-TOF MS database libraries. In our study, MALDI-TOF MS was used for identification and cluster analysis purposes. Before the COVID-19 pandemic, *C. auris* was

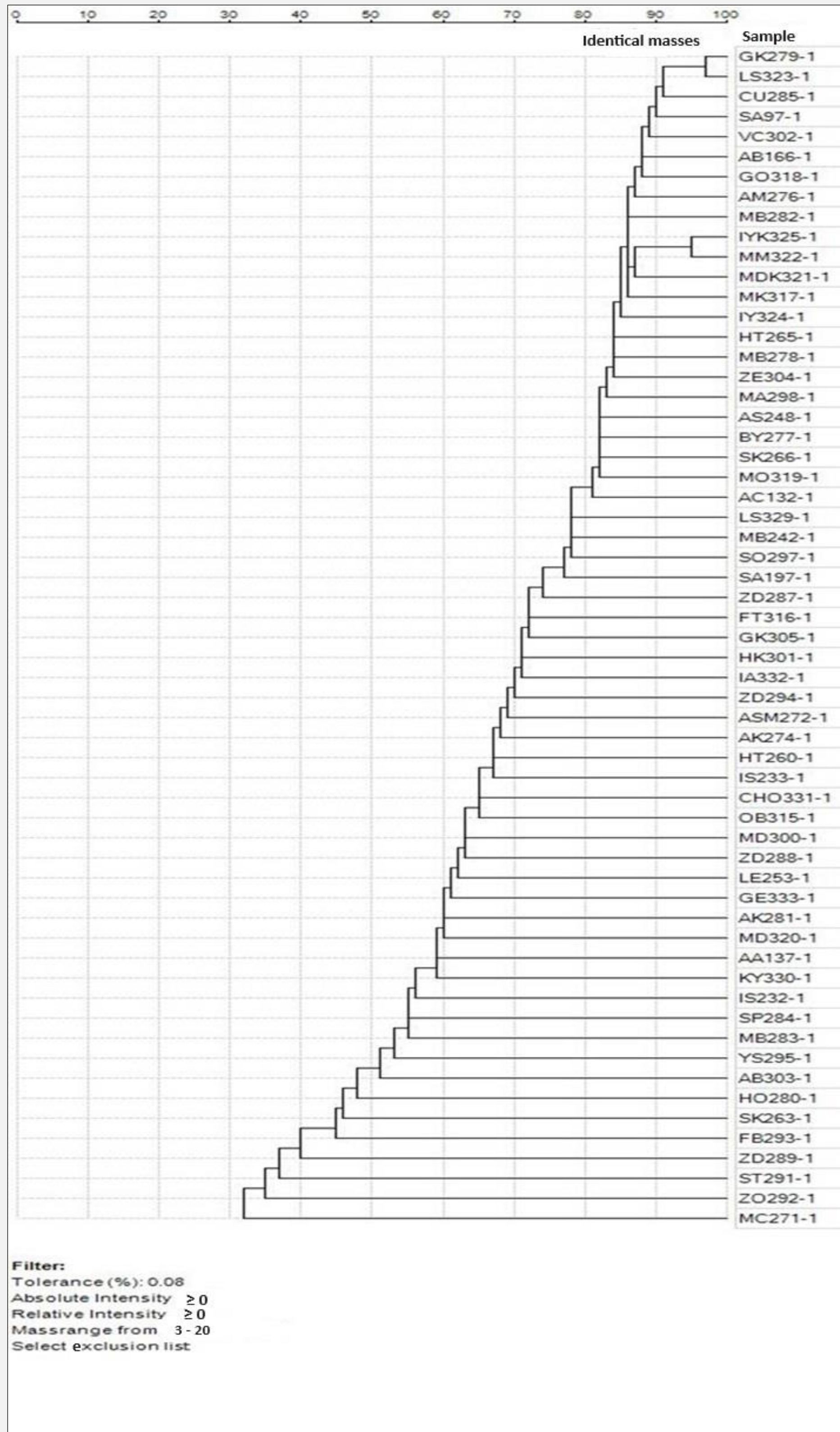

Figure 2. Distribution of *C. auris* strains in the MALDI-TOF MS dendrogram.

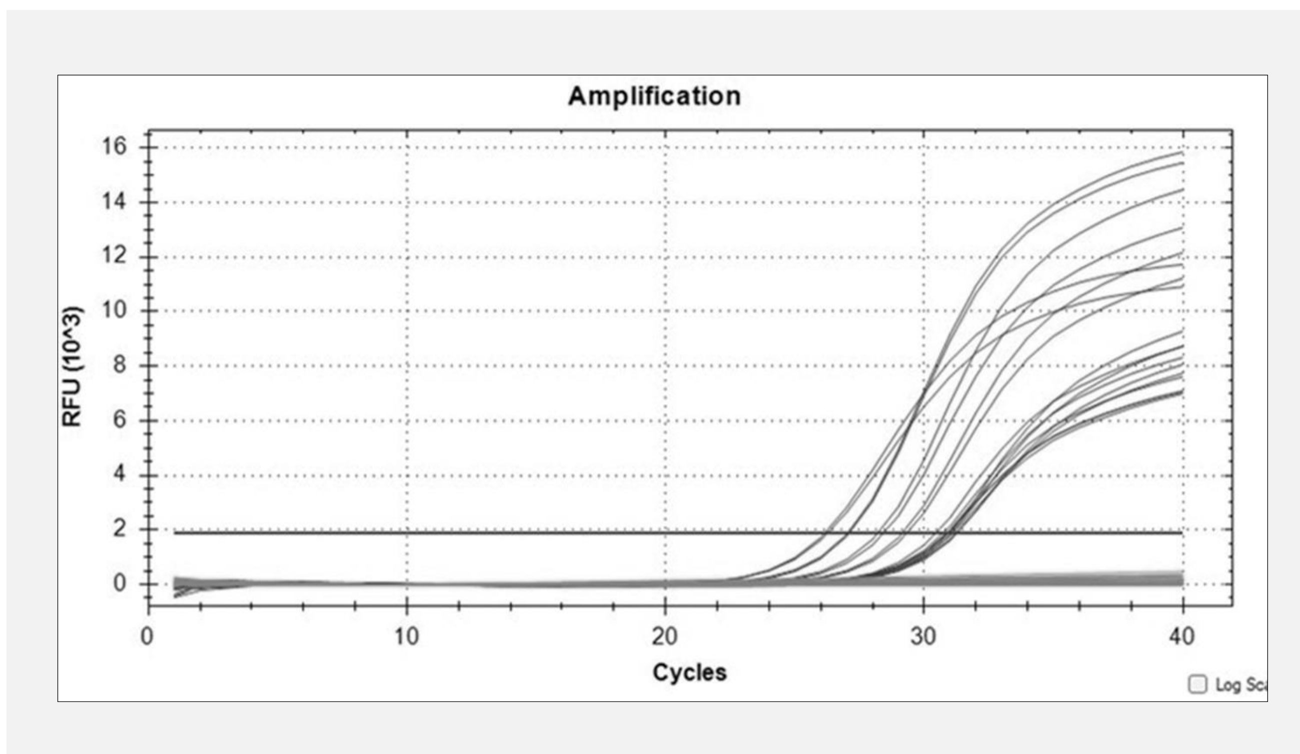

Figure 3. Clade 1 amplification curves.

not isolated in our laboratory. The first isolation emerging after the pandemic and the increase in the number of isolations during this period caught our attention and led us to consider its association with the pandemic. As mentioned before (15), it is thought that, by its very nature, yeast may increase after natural disasters. During the pandemic, people were in quarantine and the altered profile of hospitalized patients may have triggered changes in the flora, leading to infection.

The reasons for the emergence of *C. auris* are unknown, but climate change and global warming have been suggested to be significant. It has adapted to the environment outside the human host by becoming thermally adapted and salt tolerant. It can survive in wetlands, colonize amphibians, and spread through ecosystem via intermediate hosts such as birds. Excessive use of antifungal agents in both agriculture and humans has contributed to the development of antifungal resistance in *C. auris* [1,16].

In our study, 38 (65%) of the *C. auris* strains were isolated from blood, 13 (22%) from urine, 4 (7%) from tracheal aspirates, 2 (3%) from groin samples, and 1 (2%) from a wound sample. It is known that *C. auris* can colonize in patients for long periods. In one report, it was indicated that the average time between the first and last positive urine cultures for *C. auris* was 49.5 days [17]. This persistence poses a risk of transmission among immunocompromised patients [18].

The U.S. Centers for Disease Control and Prevention (CDC) issued a warning in 2023 about *C. auris* rapidly spreading in healthcare facilities in the USA, constituting an "urgent antimicrobial resistance threat" [19]. It has been reported that the increase in patients in intensive care units during the COVID-19 pandemic increases colonization of COVID-19 patients with *C. auris* and subsequently increases the risk of candidemia. *C. auris* can pose a greater threat in superinfections [1]. In our country, and also in our study, the first isolate was detected during the COVID-19 pandemic. In this study, sepsis agents were mostly isolated from blood cultures. *C. auris* mostly affects patients with underlying serious medical problems requiring complex medical care [20]. As we observed, the samples were found in critical patients such as those in intensive care and palliative care, as mentioned in the literature [1]. It was not detected in community-acquired infections but only in two patients admitted to the intensive care unit through screening of a groin swab sample. Currently, three main classes of antifungal drugs are approved for systemic use: azoles, polyenes, and echinocandins [20]. The breakpoint that can be used for *C. auris* strains has not been determined in CLSI and EUCAST, so in this study, an evaluation was made according to the tentative breakpoint values of the CDC. The sensitivity threshold values detected by the CDC are: Fluconazole ≥ 32 µg/mL, Amphotericin B ≥ 2 µg/mL, Caspofungin ≥ 2 µg/mL, Anidulafungin, and Micafungin ≥ 4 µg/Ml [20]. In a study conducted in the USA, about 90% of the

C. auris isolates were reported to be resistant to Fluconazole, about 30% were resistant to Amphotericin B, and less than 5% were resistant to Echinocandins [20]. In our study, azole resistance was detected at 84%, similar to the USA data, and high Amphotericin B resistance was detected at 60%. In another study in the U.S., more than 99% of the *C. auris* isolates were shown to be resistant to Fluconazole, nearly two-thirds were resistant to Amphotericin B, and roughly 4% were resistant to Echinocandins [21].

Although Micafungin's *in vitro* activity seems to outperform that of Caspofungin, the clinical significance of this observation remains uncertain [22].

C. auris strains were not isolated from the same clinic or hospital. It was isolated from samples coming from 5 different hospitals affiliated with our laboratory. Therefore, we did not think that it was due to nosocomial infection. However, we performed dendogram and cladistic analysis with the thought that there might be similarities between the strains.

It has been reported that resistance levels vary significantly among clades [19]. MICs to Amphotericin B (polyene) have been reported to increase in various studies and develop during the time that patients are on treatment [1,23], and resistance to Echinocandins has emerged in some countries [24].

Invasive infections are mainly caused by clade I, III, and IV isolates, whereas clade II and V isolates mainly cause otitis and are usually susceptible to antifungal drugs. Genomic analysis has shown that clade I and clade IV isolates develop resistance to Fluconazole quite readily [25]. The widespread use of antifungals to preserve crops may select for azole-resistant *C. auris* isolates that may then spread globally [1]. The differences between these clades suggest virulence-related variations. Resistance properties to azoles and polyenes among clades occur due to single nucleotide polymorphisms (SNPs) [7]. Identification of common clades by studies may guide the choice of antifungal treatment [12], as previously reported in our country [26], and the strains detected in our study were identified as clade 1. Clade 1 has been reported from South Asia, such as Kuwait, India, Pakistan, Malaysia, as well as from Germany, USA, Italy, and the Netherlands [27-30,21]. As shown in our study, in parallel with the literature, Clade I strains are resistant and multi-resistant isolates [28,31-33]. *C. auris* shows persistent colonization of human skin and abiotic surfaces in healthcare settings, facilitating inter- and intra-hospital clonal transmission that causes large outbreaks in healthcare facilities [1]. Cluster analysis based on MALDI-TOF spectrum peaks shows the relationship between isolates and clonal transmission [34]. The MALDI-TOF clustering applied to the *C. auris* strains causing nosocomial infections within the same hospital or between close distance hospitals can confirm the way of transmission [34]. In the hierarchical clustering analysis, 9 strains selected from those positioned as the closest, intermediate, and most distant strains to each other were all determined as clade 1. While it is known which clades the *C. auris* strains isolated from six continents originate from [1], epidemiological data on the phenotypic proximity of the strains isolated in our region are limited. There is a need to continue epidemiological studies on this issue.

CONCLUSION

We performed cladistic analysis on 9 strains with different similarities determined by hierarchical cluster analysis. We found that the strains in different proximity belonged to geographical clade 1 and concluded that all of the strains belonged to the same clade in terms of cost-effectiveness. Through antifungal susceptibility testing in our region, we found that resistance to azoles was close to CDC data, however, Amphotericin B resistance was high. We concluded that the reason for such a high Amphotericin B resistance in our region should be investigated in the light of more comprehensive data.

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Ethical Approval:
The study was approved by the local Ethics Committee of Kanuni Sultan Suleyman Training and Research Hospital (ethics committee no.: KAEK/2023.12.163). The study conforms to the Helsinki declaration.

Declaration of Interest:
The authors declare that they have no conflicts of interest.

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