Antifungal susceptibility testing, reporting and antifungal resistance: current status

Antifungal duyarlılık testleri, raporlama ve antifungal direnç: güncel durum

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ABSTRACT

Appropriate early treatment is crucial for prognosis in invasive fungal infections (IFIs). Antimicrobial susceptibility has generally an important role for treatment options and clinical outcome. "The European Committee on Antimicrobial Susceptibility Testing (EUCAST)" and "The Clinical and Laboratory Standards Institute (CLSI)" defined standard procedures and recommendations on interpretations of minimum inhibitory concentrations (MICs). However, they do not include epidemiological cut-off values (ECOFFs) and/or clinical breakpoints (CBPs) for every fungi and antifungal agent, so only MIC values can be shared to guide clinicians. Microbiological resistance is determined by interpreting the *in vitro* MICs with comparison of CBPs. There are many mechanisms that lead to antifungal resistance (AFR). There are increasing trends in fluconazole and echinocandin resistance for yeasts and in triazole resistance for molds. Although clinical reflections of these high MICs are sometimes very obvious, there is insufficient data to show in every fungi. Clinical resistance is the event that an infection does not resolve for various reasons despite appropriate treatment, and can be attributed to many

ÖZET

Invazif fungal enfeksiyonlarda (IFE) erken tanı ve tedavi prognoz için çok kritiktir. Antimikrobiyal duyarlılık testleri, genel olarak tedavi seçenekleri ve klinik prognoz açısından önemli bir role sahiptir. "Avrupa Antimikrobiyal Duyarlılık Testleri Komitesi (EUCAST)" ve "Klinik ve laboratuvar Standartları Enstitüsü (CLSI)" minimum inhibitör konsantrasyonların (MİK) yorumlanması için standart prosedürler ve yöntemleri belirlemişlerdir. Ancak, her mantar ve antifungal için epidemiyolojik eşik değer (EED) ve/veya klinik eşik değer (KED) tanımlanmamıştır, bu nedenle klinisyenleri yönlendirebilmek adına sadece MİK değerleri raporlanabilir. Mikrobiyolojik direnç, *in vitro* MİK değerlerinin KED verileri ile yorumlanması ile belirlenir. Antifungal dirence (AFD) yol açan çok sayıda mekanizma bulunmaktadır. Mayalarda flukonazol ve ekinokandinlere, küflerde ise triazollere dirençte bir artış eğilimi söz konusudur. Her ne kadar bazı durumlarda yüksek MİK değerleri ile klinik tablo doğrudan ilişki gösterse de her mantar için bu durum gösterilememektedir. Klinik direnç, doğru tedaviye rağmen, enfeksiyon tablosunun çeşitli başka sebeplerle düzelmemesi olayıdır ve birçok nedene bağlanabilir. Bu nedenle, antifungallere duyarlı bir

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reasons. Thus, every infection caused by susceptible organism is not always successfully treated, every infection caused by resistant organism is not always a failure. The aim of this review is to create an overall perspective to antifungal susceptibility testing and notify current condition of AFR worldwide and in our country. As IFIs show epidemiological changes and become more frequently recognized, studies on the use of antifungals have also increased, while AFR has come to the fore as one of the current problems. With *Candida auris*, it is clear that it is necessary to put an end to the relative "ignorance of fungi".

Key Words: Antifungal resistance, invasive fungal infections, candidiasis, epidemiologic cut-off value, clinical breakpoint

mikroorganizmanın oluşturduğu her enfeksiyon başarı ile tedavi edilemez, öte yandan dirençli organizma ile oluşan her enfeksiyonda da terapötik başarısızlık olmaz. Bu derlemenin amacı; antifungal duyarlılık testleri konusunda genel bir bakış sunmak ve dünyadaki ve ülkemizdeki güncel AFD durumunu tartışmaktır. IFE'ler için bir epidemiyolojik değişim söz konusudur ve bu enfeksiyonlarla daha sık karşılaşılmaktadır. Buna bağlı olarak da, antifungaller ile ilgili çalışmalar da artmış, öte yandan AFD sorunu da gündeme oturmuştur. *Candida auris* ile birlikte görülmüştür ki, mantarların görece "göz ardı edilmesine" bir son verilmelidir.

Anahtar Kelimeler: Antifungal direnç, invazif fungal enfeksiyonlar, kandidiyaz, epidemiyolojik eşik değeri, klinik eşik değeri

INTRODUCTION

Appropriate early treatment is crucial for prognosis in invasive fungal infections (IFIs) (1). For this reason, many organizations, especially The European Society of Clinical Microbiology and Infectious Diseases (ESCMID) have published various guidelines (2). In fact, joint scoring systems have been developed with the cooperation of several organizations to provide a practical approach to IFI cases (3,4). The most important problems are the processes of detecting and isolating the infectious agent, defining to the species level, and performing the antifungal susceptibility tests (AFSTs), that have long turnaround time periods.

Antifungal Susceptibility Tests

Antifungal susceptibility tests are recommended in case of i. isolates from sterile body fluids, ii. isolates with potential of antifungal resistance (AFR), iii. isolates that are rarely encountered, and iv. particular clinical requests due to a valid reason (such as treatment failure) (5-8) (Table 1). Definition of the minimum inhibitory concentrations (MICs) is not enough to evaluate the isolate, but they should also be interpreted according to the standards of "The European Committee on Antimicrobial Susceptibility Testing (EUCAST)" or "The Clinical and Laboratory Standards Institute (CLSI)". Epidemiological cut-off values (ECOFFs) and clinical breakpoints (CBPs) were determined for fungi and antifungals (9-11). ECOFFs are obtained by forming a normal distribution curve following the studies of many different strains from different geographical regions in many centers with the same method. CBPs can be defined with addition of pharmacokinetic-pharmacodynamic animal and human studies, Monte-Carlo simulation, and findings of clinical studies. In other words, while ECOFF only indicates whether the microorganism harbours an adaptive/acquired resistance mechanism, CBP actually gives data on whether therapeutic success can be achieved (12).

Table 1. Recommendations for routine mycology (adapted from references 8 and 12)

Method	Recommendation
Routine	Species level identification for strains isolated from sterile and deep infection sites
	Species level identification of *Aspergillus* and genus level identification for other molds
	Even if it is not recommended to make routine ssusceptibility tests for molds, four-well azole-containing agar screening test is advised for *A. fumigatus* complex
	Treatment according to to recommendations of international guidelines (like ESCMID)
	Report susceptibility for intrinsic resistance (IR) without performing
	Fluconazole, voriconazole and echinocandin susceptibility tests for yeasts isolated from sterile and deep infection sites
Treatment Unresponsive IFI	Amphotericin B susceptibility test
	Combination treatment
	Search for reasons which could lead to clinical resistance (e.g. invasive catheters)
Rare isolates	Susceptibility test possible but only MIC and for some species ECOFF values could be reported. Clinicians should be informed about WT, non-WT terminology.

In EUCAST standards, a limited number of fungi have the threshold values, and wild type (WT), non-wild-type (non-WT), susceptible (S), intermediate (I) and resistant (R) categories are determined. EUCAST considers only the broth microdilution (BMD) technique as the reference method and has not included caspofungin testing. In CLSI standards, there is also a Susceptible Dose-Dependent (SDD) definition in addition to other categories. CLSI accepts both BMD and disk diffusion (DD) as the reference methods. In molds, the concept of minimum effector concentration (MEC) is used instead of MIC, which's evaluation and interpretation methods are different (9-11).

In routine AFST, it is recommended to study fluconazole, voriconazole and an echinocandin (micafungin or anidulafungin) for yeasts and amphotericin B (AmB) when necessary (6). However, CLSI and EUCAST standards do not have ECOFFs and/or CBPs for every yeast and every antifungal agent. Therefore, it is not possible to interpret some MIC results. For example, for *Candida krusei*, EUCAST gave only ECOFF, except for anidulafungin and AmB. Again, EUCAST for *Candida tropicalis* did not share CBP data for micafungin. For *Candida kefyr*, there is no CBP data for either EUCAST or CLSI, and ECOFF data is very limited. Since *Candida famata* is a rare isolate, it is not included in both standards, only the MIC value can be provided for this organism. Although caspofungin is included in the CLSI standards, it is not generally recommended if another echinocandin, such as micafungin, can be studied due to interlaboratory variations (9,10,13). For *Cryptococcus* species, CLSI has determined genotype-

specific ECOFFs, while EUCAST has published species-specific values. Recently, EUCAST has also suggested ECOFF values for *Fusarium* species (13). In addition to all these, particular attention should be paid to the epidemiological data of the country of origin during reporting. Abnormal /unprecedented/unique organisms with resistance profiles observed should be sent to reference laboratories. For example, *Candida glabrata* complex, which is phenotypically resistant to echinocandins and whose molecular resistance was also confirmed, has been recently reported (14). Again, voriconazole and echinocandin resistance in *Candida albicans* is very rare.

Broth microdilution (BMD) method takes a long time and is expensive, its plates have a short lifespan (six months or less at -70°C) and it requires serious experience. Its routine application is barely possible for most laboratories, and easier and more practical methods are required. So far, many methods have been tried, such as spectrophotometric devices (VITEK 2 Yeast AST, bioMérieux, Marcy-l'Étoile, France), BMD+colorimetric kits (The Sensititre YeastOne – Thermo Scientific, Waltham MA, USA; Micronaut AM – Merlin Diagnostika, Berlin, Germany) and gradient strip tests (ETEST – bioMérieux, Marcy-l'Étoile, France; MIC Test Strip - Liofilchem Srl. Roseto degli Abruzzi, Italy). Also, studies are carried out to examine antifungal susceptibility with "matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonics GmbH; Co. KG, Bremen, Germany; VITEK®MS, bioMérieux, Marcy-l'Étoile, France)". However, there are serious reliability issues with these tests. Actually, ECOFF and CBP data are method specific, and whether the threshold values determined for CLSI or EUCAST for BMD can be used in the interpretation of the results obtained by other methods have been the subject of many studies. CLSI standards are generally recommended with VITEK 2 Yeast AST (bioMérieux, Marcy-l'Étoile, France) and good agreement was observed. On the other hand, it has been reported that there may be problems in fluconazole analysis, especially for *C. glabrata* complex and *C. kefyr*. In addition, interlaboratory variations have been reported and the narrow MIC range for AmB causes problems. It has been reported to have false-resistant results for some strains and antifungals (such as *Candida auris*) (5). The SensititreYeastOne (SYO) (Thermo Scientific, Waltham MA, USA) is a BMD-based method containing the alamarBlue indicator. This technique, which is in good harmony with CLSI, creates advantages such as long shelf life and easy usability. It should be noted that the lowest categorical agreement was observed with the reference methods for *C. glabrata* complex and *C. tropicalis*, although the researchers reported that they observed less than 1% major and minor errors. Micronaut AM (Merlin Diagnostika, Berlin, Germany) is also a SYO-like kit based on EUCAST. However, this technique still needs multicenter studies involving many strains. When the gradient strip method was performed with RPMI 1640 agar with 2% glucose, after 24 to 48 hours of incubation, azole and echinocandin (except caspofungin) in *Candida* isolates showed a 90% or more agreement with reference methods. Researchers especially stated that they did not encounter "very major error (susceptible result to resistant strain)" (5,7,15). Although such promising results have been observed, the authorities are still hesitant about the interpretation with ECOFF and CBP values in the routine laboratory, since these values are method-specific and significant variations were observed in interlaboratory studies. Therefore, it is essential to use reference methods (12).

Recently, EUCAST recommended the four-well azole-containing agar screening method (azole-agar screening) for *Aspergillus fumigatus* complex. This test is a method depending on whether there is a growth after inoculation of certain inoculum isolates on RPMI 1640 agar plates containing itraconazole, posaconazole and voriconazole and incubation for 48 hours. However, as the name suggests, it is a screening test, since resistant strains should be tested with the reference method for confirmation (15,16). The

performance of this test is relatively poor with sibling species (complex members other than *A. fumigatus sensu stricto*) , also called cryptic strain (15,17). There are studies indicating same method to be used for echinocandins in *Aspergillus* species, but it has not been included in the guidelines yet (18).

Identification of fungi with the MALDI-TOF MS device has entered routine laboratory use. Since it is basically a mass spectrophotometry, studies have been carried out that it can also be used in the determination of the AFR profile. The method is based on the examination of the spectra of fungi exposed to the antifungal agent at different dilutions and their interpretation according to the minimum profile change concentration (MPCC). However, for now, there are problems of reproducibility, standardization, validation and profile library (5,7,13,15).

Culture independent molecular techniques are also in the agenda for the identification of fungi. SeptiFast (Roche, Basel, Switzerland) and T2 *Candida* system (T2 Biosystems, Lexington, MA, USA) for the detection and identification of *Candida* species, and AsperGenius (PathoNostics, Maastricht, Netherlands) and MycoGENIE (Ademtech, Pessac, France) kits for *Aspergillus* were developed. However, these kits do not comment on antifungal susceptibility. Molecular investigation of azole resistance in *Candida* species is challenging, as multiple mechanisms for resistance operate and their genetic origins are different. The problem in *Aspergillus* is that in only 30% of the azole-resistant strains the resistance mechanisms have been elucidated. In this context, searching for a resistance mutation for *A. fumigatus* complex by AsperGenius (PathoNostics, Maastricht, Netherlands) and MycoGENIE (Ademtech, Pessac, France) kits may provide some clinical benefit, but it is not yet recommended in the routine laboratory. On the other hand, echinocandin resistance in *Candida* species is generally based on the *FKS* mutations. Especially for *C. glabrata* complex, the demonstration of *FKS* mutations in cases of therapeutic failure may indicate that the strain may be resistant to echinocandins, even in lack of any MIC data (7,12).

Clinical resistance can be encountered and therapeutic failure may occur even in such cases of susceptible MICs (90-60 rule) (12,15,19). Here it is necessary to explain the concepts of resistance. Microbiological resistance and clinical resistance are defined as different concepts. Microbiological resistance is determined by interpreting the MIC value with comparison of CBPs. Two concepts have also come into play for microbiological resistance; intrinsic resistance (IR) and acquired resistance. The terminology of IR is defined as the resistance of the microorganism to an antifungal drug due to its inherent functional or structural characteristics (lack of drug target, inability of the drug to penetrate the cell wall/membrane, etc.). This type of resistance is seen in all strains of that species and is independent of exposure to the antifungal drug. Acquired resistance is seen in some strains of that species that are normally susceptible to the antifungal drug, usually following exposure (clinical practice or environmental exposure). These two resistance profiles generally use the same molecular mechanisms. The IR status of fungi, which are frequently seen as clinical agents, are summarized in Table 2, whereas acquired resistance can be highly variable within the species. Clinical resistance, on the other hand, is the event that an infection does not resolve for various reasons despite appropriate treatment, and can be attributed to many reasons (Table 3) (19,20). In other words, while microbiological resistance is a laboratory terminology, clinical resistance is based on a clinical condition. Of course, within this concept, the question of compatibility of microbiological and clinical resistance, how resistance affect clinical success and at which MIC values comes into play.

Recommendations for reporting susceptibility by considering both the type of microorganism, the pharmacological properties of the antifungal, and the type/location of infection are presented in Table 4 (6).

Table 2. Intrinsic Resistance (adapted from references 6 and 8)

Organism/Antifungal	Fluconazole	Isavuconazole	Itraconazole	Posaconazole	Voriconazole	Echinocandins[1]	Flucytosine	Amphotericin B
Candida krusei	IR							
Candida lusitaniae								*
Cryptococcus spp.						IR		
Rhodotorula spp.						IR		
Trichosporon spp.						IR		
Order of *Mucorales*	IR				IR	IR**		
L. prolificans	IR	UI	IR	IR	***	UI		UI
Fusarium spp.						UI		
Purpureocillium lilacinum								UI
Aspergillus terreus complex								NR****
Aspergillus spp.	IR						NR*****	

IR: Intrinsic Resistance; NR: No Reporting; UI: Under Investigation; [1]Micafungin, Anidulafungin and Caspofungin

* *C.lusitaniae* is not intrinsically resistant to AmB, but resistance may occur during treatment.

** Order of *Mucorales* is accepted as intrinsically resistant to echinocandins in vitro. However, it can be effective in combination therapies. It is not recommended as a monotherapy agent.

*** *L. prolificans* is intrinsically resistant to azoles except voriconazole.

**** MIC values do not correlate with clinical outcome, AFST is not recommended.

***** For *Aspergillus* spp. flucytosine resistance cannot be detected due to pH issues in in vitro tests. Flucytosine can be effective in combination therapies.

Table 3. Factors Which Cause Clinical Resistance (adapted from references 49 and 50)

Level of immunosuppression	Directly related to immunity: Neutropenia, HIV etc.
Microorganism load	Onset of treatment and number of microorganisms at the site of infection
Acquired increase in virulance	Although AFR and virulance are inversely correlated, there is increased virulance of *C. glabrata* complex
Pharmacodynamic/ Pharmacokinetic properties	PD indexes, concentration in infection sites, presystemic elimination etc
Site of infection	Drug penetration, biofilm, link with AFR
Underlying diseases	Comorbidities
Duration of treatment	a. Incompatibility of clinician and/or patient with long term treatment protocols; b. Clinicians' perception of culture positivity as AFR despite successful antifungal treatment
Antibiotics ??	Some antibiotics' promoting effect on fungal growth?

Table 4. Recommendations for routine mycology (adapted from references 6, 8 and 13)

Antifungal	Specimen	Recommendation
Amphotericin B	All specimens	No limitation
Echinocandins	Urine	Not report echinocandins. Passage of echinocandins to urine below 1%.
Echinocandins	Ocular samples (cornea, aqueous and vitrous fluid)	Not report echinocandins. Penetration of echinocandins to ocular tissue is highly limited.
Echinocandins	Central Nervous System (CNS) Specimens (Tissue, Abscess, CSF)	Can be reported. Passage of echinocandins to CNS and CSF is weak, but they could reach to effective concentrations against *Candida*.
Azoles	Urine	Only test and report fluconazole
Azoles	Ocular specimens (Cornea, aqueous and vitrous fluid)	Report fluconazole and voriconazole
Azoles	CNS Specimen (Tissue, Abscess, CSF)	Report fluconazole and voriconazole

Antifungal Susceptibility and Clinical Reflections

The relationship between clinical prognosis and AFR is not always correlated. "90-60" rule (probability of an infection caused by an antimicrobial-susceptible strain to respond to the correct treatment is 90%, the probability of response to treatment in an infection caused by a resistant strain is approximately 60%.)" is based on a large-scale study (15,19). On the other hand, some studies have found a significant relationship between outcome and microbiological resistance in certain strains;

a. Although the relationship between caspofungin MIC level and prognosis is controversial for *Candida* species (it should be noted that EUCAST and CLSI do not recommend the use of caspofungin for testing), previous echinocandin exposure, presence of *FKS* mutation, and echinocandin MIC levels are significantly meaningful especially for *C. glabrata* complex. It has been noted that the most serious adverse effect on prognosis was that the strain showed echinocandin resistance in addition to previous echinocandin exposure. It seems clinically difficult to make an interpretation based on the MIC level alone (12). The issue of echinocandin resistance for *Aspergillus* species is not clear, the studies are very limited, and the place of echinocandins in the treatment of invasive aspergillosis (IA) is mostly in the form of combination therapies (21).

b. Although there are many studies on *Candida* infections and azole antifungals, it has not been possible to directly demonstrate the prognostic effect of azole resistance. Authorities draw attention to the necessity of randomized controlled studies on the subject (12). On the other hand, studies indicate that fluconazole MIC values are directly related to therapeutic success (22). The recommendation derived from these data is that this antifungal should not be preferred in infections of strains that

are generally resistant or have high MIC values. In *Aspergillus* species, the relationship between azole resistance and prognosis is much more evident; such that there are recommendations on this subject even in the ESCMID guidelines (21). Azole resistance of environmental origin (due to exposure to pesticides) has become serious in *Aspergillus* species, especially in some countries (such as the Benelux region). Therefore, in countries where the incidence of azole resistance is more than 10%, experts recommend adding an echinocandin to the initial voriconazole therapy (12). The ESCMID guideline strongly recommends the azole agar screening test and the species-level determination of clinical *Aspergillus* strains for routine laboratories, with a particular warning about cryptic species (21).

c. The issue of cryptococcosis is still obscure. Current treatment approach is with AmB (flucytosine may be added to this). Although it is stated that "step-down" can be realized later according to the susceptibility test results, the data on outcome with fluconazole MICs are very limited (12,23).

d. Studies for other rare yeasts and molds are scarce and although there are not enough data, the general principle is to pay attention to IR. Many organizations, especially ESCMID, have already published their guides (23,24).

Mechanisms of Antifungal Resistance

Azole Resistance: Azoles target the enzyme lanosterol 14-alpha demethylase, which is dependent on cytochrome P450 in the synthesis of ergosterol. The synthesis of this enzyme is controlled by *ERG11* genes in yeasts and *Cyp51* genes in molds. Disruption of ergosterol synthesis in the cell membrane results in fungistatic effects in yeasts and fungicidal effects in molds. Azole resistance is basically based on three main mechanisms: i) up-regulation of the coding of the mentioned enzyme, ii) changes in the azole target site, iii) up-regulation of efflux pumps. Although the dominant mechanism varies according to the species, more than one mechanism may be active in one strain (25).

In *Candida* species, these mechanisms include point mutations in the *ERG11* sequence, mutations in the regulatory domains of *ERG11* transcription (Upc2) and its associated upregulations (gain-on-function mutations - GOF) and/or Cdr1 and Cdr2 (ABC - "ATP" binding cassette type carrier), and upregulated by GOF mutations (in Tac1 and Mrr1 transcription factors) in efflux pumps. In general, changes in efflux pumps in *Candida* species are thought to be the most rapidly developing resistance mechanism after azole exposure. *ERG11* mutations are mostly concentrated in three "hot-spot (HS)" areas of the enzyme and for *C. albicans*, is especially effective on fluconazole MICs, itraconazole and voriconazole are not much affected by this mutation. The only exception is the Y132F substitution, where all three triazole MICs are affected. In addition to GOF mutations in regulatory domains in *ERG11*, there is chromosomal aneuploidy (chromosome 5). There are also specific cases for *C. glabrata* complex. The mutation of the *MSH2* gene, which encodes the protein involved in the DNA repair mechanism, has been found in most of the resistant strains, but a direct link with AFR has not been established yet. In the case of inhibition of ergosterol synthesis, *C. glabrata* complex also has a unique tolerance mechanism, that it compensate for the loss of its own ergostrol by ingesting serum cholesterol *in vivo*. Although this ingestion also exists in *C. albicans*, the mechanism of *C. glabrata* complex also works under anaerobic conditions and works more rapidly. In *C. parapsilosis* complex, the dominant resistance mechanism is the *ERG11* mutation (Y132F and K143R substitutions). Cross-resistance to other azoles is seen in 60% of fluconazole-resistant *C. parapsilosis* complex strains. *C. krusei* is inherently resistant to fluconazole (its mechanism has not yet been fully elucidated) but is mostly susceptible to other azoles. The major cause of resistance to other azoles is the intense azole exposure of the microorganism due to fluconazole prophylaxis. The high azole MICs seen in *Candida guillermondii* complex are also thought to

be due to the *ERG11* mutation. There is no or very limited information on other *Candida* species showing high MIC levels (such as *Candida norvegensis*, *Candida inconspicua*, *Candida lipolytica*). It should be noted here that the main cause of fluconazole resistance in *Cryptococcus* species is *ERG11* mutations (20,25,26).

Fluconazole should not be used for mold infections and it has no *in vivo* activity. Two problems stand out with regard to azoles in *Aspergillus* species; i) data on the epidemiology of cryptic species are scarce, but these species may exhibit different resistance profiles, ii) due to environmental azole exposure (such as pesticides), azole-resistant *Aspergillus* rates of up to 30% have been observed in various regions of the world. EUCAST and CLSI have identified a limited number of ECOFF and CBPs related to molds. Warnings regarding cryptic species can be found in ESCMID's directory. Azole resistance in *Aspergillus* species is caused by polymorphisms in CYP51 protein and changes in azole target, increase in target enzyme coding and synthesis, and efflux pumps. In addition to these, biofilm and enzymatic degradation methods of the drug are also available. The CYP51 enzyme encoded by the *CYP51* gene is essential in *Aspergillus* azole resistance. *A. fumigatus* complex, *A. nidulans* and *A. niger* complex carry two *CYP51* paralogs (A and B), while *A. flavus* complex carries three paralogs (additional C). Acquired resistance is largely due to the *CYP51A* mutation and may be accompanied by *CYP51B*. Single *CYP51B* mutation is very rare and its association with resistance has not been demonstrated. *A. fumigatus* complex showing azole resistance but containing wild-type *CYP51A* is very rare. In fact, there are especially *A. flavus* complex strains that do not have a *CYP51* mutation but are evaluated as non-WT. Therefore, phenotypic tests are more valuable than molecular tests, and ESCMID/EUCAST or CLSI criteria should be followed in terms of both AFST and treatment. In addition to mutations, the "*CYP51A* promoter tandem repeat (TR)" status causes up-regulation of the *CYP51A* gene, leading to its increased coding, which brings with it azole resistance. In this mechanism, TR34/L98H and TR46/Y121F/T289A are the observed changes. In efflux pumps (ATP Binding Cassette – ABC and Major Facilitator Superfamily – MFS), very few genes have been shown to be related. It is thought that *cdr1* (*abcB*) from ABC family for *A. fumigatus* and *A. flavus* complex, and *mdrA*, *mfsA*, *mfsB* and *mfsC* from MFS family for *A. fumigatus* complex are thought to be related (21,25,26).

Echinocandin Resistance: Echinocandins (micafungin, caspofungin, anidulafungin, and rezafungin) block glucan synthesis in the fungal cell wall structure by targeting the (1–3)-ß-D-glucan synthase enzyme non-competitively. Acquired resistance in *Candida* species is low (less than 3%). The exception is *C. glabrata* complex, and its resistance is reported to be increasing especially in the world. Echinocandin resistance is mainly caused by three mechanisms; i) stress response pathways (increased chitin synthesis), ii) acquired mutations in the *FKS* gene encoding the (1–3)-ß-D-glucan synthase enzyme, iii) inherent *FKS* variations (*C. parapsilosis* complex and *C. guilliermondii* complex; MIC levels higher than those of acquired mutations). Resistance mutations in *C. albicans* and many other *Candida* species occur in the "hot spot" areas of *FKS1*. In addition, or singularly, *FKS2* mutations are observed in *C. glabrata* complex. The mutation disrupts the drug affinity of the target enzyme and increases the MIC levels. These heterozygous mutations in diploid *Candida* species pose a serious "cost & fitness" problem, which may explain why resistant strains are rare. It should be noted here that *C. glabrata* complex is haploid and echinocandin-resistant *C. glabrata* complex strains can even show cross-resistance with polyene and azole group antifungals. Although the resistance mechanisms of *C. auris* have not been fully elucidated, *FKS1* mutations have been shown (20,26,27).

Echinocandin resistance has also been observed in *Aspergillus* species, but *FKS* mutation has not been demonstrated in these strains. As a matter of fact,

although it was shown that *FKS1* was encoded in species such as the order of *Mucorales* and *Fusarium solani* complex, which are intrinsically resistant to echinocandins, there was not mutations. This indicates that resistance develops in these molds by a mechanism other than *FKS*, which needs further studies (26).

Polyene Resistance: AmB is a fungicidal drug, resistance is rare, as its resistance creates a serious "cost & fitness" problem. *C. guilliermondii* complex, *A. terreus* complex, some species of order of *Mucorales* and most *Fusarium* species are inherently resistant. It has been reported that some members of the *C. lusitaniae* and *C. haemulonii* complex show rapid resistance. Although it has been stated that *ERG11*, *ERG3*, *ERG2*, *ERG5* mutations which are heterozygous for *C. albicans* and *ERG2* and *ERG6* mutations in *C. glabrata* complex cause AmB cross-resistance, the mechanism of AmB resistance is still obscure. Such that, except for the *ERG6* mutation, all of them also have cross azole resistance. It is thought that methods of combating oxidative stress (such as "heat shock" proteins-Hsp, superoxide dismutase, catalase) are effective in *A. terreus* complex (20,26).

"Cost & Fitness", Antifungal Tolerance and Heteroresistance: Although AFR ensures the survival of the microorganism, it comes with a price. Generally, the sporulation and growth rate of the microorganism are adversely affected, which leads to a decrease in its virulence. Studies on this subject in azole resistance mostly focused on *C. albicans* and fluconazole. It is noteworthy here that the loss of "fitness" is not due to a single mechanism, but with a cumulative effect. On the contrary, azole resistance developed in *C. glabrata* complex contributes to the virulence of the microorganism. A similar situation is experienced in echinocandin resistance. While *FKS* mutant *C. albicans* loses a degree of ability in reproduction, their hyphal capacity decreases and its virulence is negatively affected, there is no change in this sense for *C. glabrata* complex. AmB causes a great level of "Cost & Fitness". AmB-resistant organisms are highly susceptible to external stressors, including oxidative stress, and lose their virulence extensively (26).

When an antifungal susceptible organism is exposed to an antifungal, the ability of some subpopulations of the microorganism to grow even more slowly in the presence of that antifungal is considered as antifungal tolerance. This subpopulation is thought to have this capability via various mechanisms such as Hsp90 and calcineurin. In fact, this situation is defined as the "trailing" effect in *in vitro* AFST. In studies focusing especially on fluconazole and *C. albicans*, it has been stated that this is not exactly defined as resistance, it is directly related to the drug concentration encountered by the microorganism, but it has a therapeutic reflection, and persistent candidemia cases are experienced in such cases (26,28).

Heteroresistance is demonstrated especially in the azole exposure of *C. neoformans* complex. The issue here is that a "reversible resistance" is observed in the subpopulation of the microorganism. This group, which shows drug resistance on azole exposure, loses its "resistance" after the exposure is ended. This ability is thought to be due to the plasticity of cryptococcal genes. Combination therapies seem to be the key to therapeutic success in infections with both tolerant and heteroresistant strains. Because of this ability of cryptococci, combined antifungal treatments have been recommended by the guidelines in cryptococcosis infections (28).

Candida species are well-known for their ability to form biofilms. Due to their ability to adhere to surfaces, they can cause manifestations such as catheter-related infections. Although (1-3)-ß-D-glucan is the key molecule in the biofilm structure, biofilm formation is a multi-mechanical event. *Candida* biofilms show severe tolerance to antifungals. Although mutations that may cause resistance have been encountered, the main mechanism is the prevention of penetration of drugs by the glucan matrix (27).

Epidemiology of Antifungal Resistance

AFST is an analysis that is laborious, expensive and unnecessary in all cases. Therefore, epidemiological data on AFR are of striking importance. On the other hand, as epidemiological studies have increased, microbiological resistance profiles that show serious variations even at the species level have been encountered (1).

Although *C. albicans* is the most common causative yeast all over the world, there are variations according to geographical areas in following rankings. *C. glabrata* complex, *C. parapsilosis* complex, *C. krusei* and *C. tropicalis* are the leading yeasts, while *Aspergillus* species lead among molds. Therefore, various studies have been conducted on AFR of these microorganisms. In general, fluconazole resistance is less than 1% for *C. albicans* and up to 11% for *C. glabrata* complex, for *C. tropicalis* (below 10%) and *C. parapsilosis* complex (2-5%), however there is an increasing trend in fluconazole resistance. *C. glabrata* complex raises the alarm all over the world in fluconazole resistance and the most serious rates are obtained from North America (10.6%). In addition, *C. parapsilosis* complex shows a similar trend in Europe and Latin America. Unlike *Candida* species, fluconazole resistance in *Cryptococcus* species is stable, however, fluconazole resistance increases up to 24% in relapsed cases (12,26,29,30).

The most serious problem in AFR is the increasing pattern of echinocandin resistance in *C. glabrata* complex and *C. krusei*. The cross-resistance of *C. glabrata* complex with azoles indicates that the treatment options are getting limited in the infections of this microorganism (26,28,29). Multidrug resistance (MDR) can also be seen in *Candida* species, that is generally with both acquired resistance and IR. MDR with singular acquired resistance is rare. *ERG3* and *ERG2* alterations may cause azole and AmB cross-resistance in *C. albicans* and *C. dubliniensis*. Interestingly, previous fluconazole treatment may be a trigger for echinocandin-resistant *C. glabrata* complex. Again, the site of infection (exposure to drug concentrations below therapeutic doses; abdominal, mucosal areas, foreign body) and biofilm formation are important parameters for the development of MDR (31).

The most prominent representative of AFR today is *C. auris*. 93%, 35% and 7% of strains are resistant to fluconazole, AmB, echinocandins, respectively. 41% of strains are resistant to two different classes of antifungals, and 4% of strains are resistant to three different classes of antifungals. It has been on the world's microbiology agenda with its colonization, ability to survive on surfaces for weeks, and high resistance to disinfectants (32). Unfortunately, the recognition of this microorganism in routine laboratories is directly related to the awareness and technical capacity of laboratory specialists, as the microorganism is misidentified even with many semi-automated/automated methods. According to the records of the US Centers for Disease Control and Prevention (CDC), *C. auris* has been reported from 47 countries as of February 2021, but this is actually thought to be higher (33). As a matter of fact, there have been consecutive notifications from Turkey (34,35). The CDC has published a recommendation guideline on when and in which cases screening programs for *C. auris* should be performed (33).

In the study of Calgin and Cetinkol (36), the resistance profile of clinical *Candida* isolates were studied with the VITEK 2 system (bioMérieux, Marcy-l'Étoile, France) and AmB, flucytosine, fluconazole, voriconazole, caspofungin and micafungin resistance were 7.3%, 10%, 9.4%, 7.3%, 2% and 6.5%, respectively. The problem here is the automated system to give false resistant results, especially in some strains and antifungals, and the rates of echinocandin and AmB resistance are very remarkable. Yenisehirli et al. (37) studied AFST with gradient strip test in non-*albicans Candida* species, and they could not detect any strains non-susceptible to echinocandins, except for two *C. tropicalis* isolates, which were found to be in intermediate zone. They also did not find a *C. parapsilosis* complex strain with azole resistance. In

another study that meta-analysed *C. albicans* studies from Turkey, the average resistance to itraconazole and voriconazole was 23.2% and 14.6%. Fluconazole resistance was reported as 9.6% and none of the cases were echinocandin-resistant (38). It is obvious that *C. parapsilosis* complex has become a prevalent problem in Turkey over time. In the multicenter study of Hilmioğlu-Polat et al. published in 2018 (39), although only *C. parasilosis* sensu stricto strains were studied, 9.4% of fluconazole resistance and 4.5% of voriconazole resistance were found, and there was not any echinocandin and AmB resistance. This was followed by fluconazole-resistant *C. parapsilosis* complex clonal spread including *ERG11* Y132F/Y132F+K143R substitutions (40) and similar results were also supported by Demirci-Duarte et al. (41) (*C. parapsilosis* complex; fluconazole resistance is 13.3%; Y132F type resistance is 71.7%) . Unfortunately, fluconazole non-susceptible strains have also been reported in *C. tropicalis* isolates (42). In another recently published study, fluconazole resistance was observed in 9.2% and itraconazole resistance in 45.8% in *C. glabrata* complex strains, while 43.4% of isolates were of the non-WT category for voriconazole (43). Considering the cross-resistance nature of *C. glabrata* complex, it has been claimed that if high fluconazole MIC levels and echinocandin *FKS* mutations are detected in coordination, it may be a prognostic factor for therapeutic failure (44). These studies show that azole treatment options are being lost in various strains for Turkey and that echinocandins are also under threat. As a matter of fact, in the large multicenter candidemia study of Arikan-Akdagli et al. (45), fluconazole resistance (7.7%) was observed in *C. parapsilosis* complex strains, but very low fluconazole resistance rates in *C. glabrata* complex strains and absence of resistant *C. tropicalis* strains were notifying. Furthermore, none of the isolates showed echinocandin resistance. On the other hand, this study was followed by the detection of *C. glabrata* complex isolates without phenotypic echinocandin resistance but with *FKS* mutation (44). Just recently, clinical *C. glabrata* complex isolates with both phenotypic echinocandin resistance and *FKS* mutations were reported (14).

Studies on the epidemiology of *Aspergillus* infections and AFR in Turkey are very limited. The largest study belongs to the recently published 12-year data of Gülmez et al. (1), indicating that *A. fumigatus* complex (50.4%) was most frequently isolated in mold in lower respiratory tract samples, as expected, followed by other *Aspergillus* species (31.3%). However, the most important finding of this study was that there was a significant decrease in the isolation rates of *A. fumigatus* complex over a 12-year period, while numbers of other *Aspergillus* species and non-*Aspergillus* molds (*Penicillium* spp., order *Mucorales*, *Scedosporium* spp., *Alternaria* spp., *Paecilomyces* spp., dematiaceous fungi and unidentified molds) increased significantly. As a matter of fact, with the intensive use of antimicrobials especially in cystic fibrosis patients and the increase in the average life span of these patients, the isolation of different types of molds from the respiratory tract has also increased significantly (46). A clinical strain showing a *CYP51* mutation (TR34/L98H) which is resistant to azole group (itraconazole, voriconazole, posaconazole) drugs from Turkey was reported in 2015 during retrospective screening of laboratory isolates (47). Also in 2018, an *A. fumigatus* complex isolate respiratory sample of a cystic fibrosis patient with phenotypic azole resistance, but without *CYP51A* mutation (48). Obviously, azole-resistant *Aspergillus* is present in Turkey, but its prevalence is obscure.

Although the resistance profile in Turkey is not generally threatening for *Candida* species, one by one resistant cases are reported. It is obvious that a national surveillance network on AFR should be established in Turkey as well. In addition, awareness on *C. auris* needs to be increased, as it has already entered our agenda. Again, there is very little data on the resistance of clinical *Aspergillus* strains in Turkey, and the relationship of resistance

in *Aspergillus* species depending on the use of fungicide should also be investigated.

In conclusion, as IFIs show epidemiological changes and become more frequently recognized, studies on the use of antifungals have also increased, while AFR has come to the fore as one of the current problems. With *C. auris*, it is clear that it is necessary to put an end to the relative "ignorance of fungi".

Phenotypic AFST methods allow the demonstration of resistance status regardless of fungal species, even with defined or not yet defined mechanisms. However, they require serious experience and expertise and in some cases, there are problems with MIC detection and accurate interpretation due to variations between laboratories. As a matter of fact, molecular methods can provide an advantage to the laboratory tests when detecting AFR. However, its place in routine laboratories is still controversial (20). Currently, other than the CLSI and EUCAST reference methods, none of the AFST methods could be recommended.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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