A Silent Threat of Emerging Resistance Based on Antifungal Susceptibility Pattern of Filamentous Fungi by Microdilution, E Test and, Disc Diffusion Method: A Critical Constructive Analysis

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Abstract
For more than two decades, antifungal susceptibility testing and interpretation haunted the medical professionals in diagnostics and management. This article mainly focuses on the three most widely used methods: broth microdilution, E test, and disc diffusion. It also focuses on the fact that clinicians should switch from empirical treatment to susceptible drugs as early as possible to combat antifungal resistance and newer mutations that horrify us every single day with poor patient outcomes. Many factors need to be taken into account during the interpretation of results but the positive side of the story is that they have been well documented in the literature. Though many methods have come up in testing antifungal susceptibility, still there is a scope for a rapid yet accurate testing modality to flourish and take the lead.

Keywords
► filamentous fungi
► broth microdilution
► BMD
► disc diffusion
► E test

Introduction
Fungal infections are the leading cause of ocular, nasal, ear, urinary tract, and various other infection sites. Fungal infections, though more common in immunocompromised individuals, need to be given special consideration as far as patient care is concerned. The slow growth of fungi is a major halt in terms of diagnosis and consequent patient prognosis as compared with dealing with bacterial infections. Antimicrobial susceptibility has a direct impact on patient outcomes. Keeping this fact in view, various studies have been included to make clinicians aware of growing resistance to various antifungal drugs. Broth microdilution (BMD) and disc diffusion (DD) methods are most widely used. E (Epsilometer) test has its advantage in terms of flexibility in choosing test medium and somewhat earlier results. With the emergence of a newer mechanism of resistance, strict and continued vigilance of accuracy of each test is the need of the hour.

Methods
Antifungal Susceptibility Testing (In Vitro)
Broth Microdilution Method
Antifungal susceptibility testing is performed against various antifungal drugs by following Clinical and Laboratory...
Standards Institute (CLSI) M38-A2 guidelines. The procedure involves dispensing the test drug mixed with Roswell Park Memorial Institute (RPMI) medium and conidial suspension in microtiter trays followed by incubation at 35°C and reading it at 24 hours (Rhizopus spp.) and 48 hours (for all other species). The minimum inhibitory concentration (MIC) end point is defined as the lowest concentration that produces prominent inhibition (~50% inhibition) of growth relative to the drug-free growth control. The final concentration of the antifungal agents ranges from 0.016 to 16 μg/mL for fluconazole, itraconazole, voriconazole, posaconazole, amphotericin B, anidulafungin, and caspofungin. For fluconosine, the concentration varies from 0.064 to 64 μg/mL.

Disc Diffusion Method
This method is documented in CLSI M51-A and is still widely used for nondermatophyte filamentous fungi. It has the advantage of being cheaper in resource-poor settings, when compared with otherwise standard and the best BMD method.

CLSI Broth Microdilution (M38-A2), E test, and CLSI Disk Diffusion (M51-A)
In a comparative study of antifungal susceptibility for Aspergillus spp. based on the E test, BMD, and DD, the E test (93.8% agreement) gave better concordant results with BMD than DD (87.5% agreement). In a study done by Pfaller et al., the E test method using RPMI agar was found to be useful for determining itraconazole susceptibilities of Aspergillus spp. and other filamentous fungi. The study highlighted many facts and succeeded in clearing many doubts in terms of determining authentic methods for antifungal susceptibility and interpretation of results. One important fact that needs to be reminded of is that the E test MIC was read as the drug concentration at the point where dense colonial growth intersected the strip, ignoring sparse subsurface hyphae at the margins.

Other Methods
Agar dilution, determination of fungicidal activity, flow cytometry, and ergosterol quantitation are other options that can help in our quest for better and reproducible results.

Mechanism of Action of Antifungal Drugs
Azole agents exert their antifungal activity by blocking the demethylation of lanosterol, thereby inhibiting ergosterol synthesis. Among various azoles, voriconazole has the broadest spectrum of activity with excellent results. The polyene agents exert their antifungal activity via binding to ergosterol in the fungal cell membrane. This disrupts cell permeability and results in rapid cell death.

Resistance Pattern and Therapeutic Options for Various Isolates
Resistance of Aspergillus fumigatus was observed to be occasionally present (11%), with voriconazole, ravuconazole, and itraconazole being most active (percentage resistant, respectively: 11, 38, and 52%). Posaconazole is an upcoming new option for refractory invasive fungal infections and coccidioidomycosis. A retrospective study reported better response with oral posaconazole than with the combination of intravenous liposomal amphotericin B plus caspofungin in refractory invasive aspergillosis.

Intrinsic Resistance: Let us Not Forget
At the Interscience Conference of Antimicrobial Agents and Chemotherapy (ICAAC) meeting (2004), a matched case–control observational study of 27 recently treated cancer patients with zygomycosis indicated that Rhizopus was the most common species involved and that the clinical strains were resistant to voriconazole. The said resistance was later emphasized in many other articles as intrinsic resistance. Similarly, Aspergillus terreus is intrinsically resistant to amphotericin B. Hence, established microbiologists as well as postgraduate residents while reporting antifungal susceptibility to the clinicians should be aware of intrinsic resistance of specific fungi to avoid in vitro and in vivo discordance of results.

Discussion
In a study done by Pfaller et al., susceptibility testing of clinical isolates was performed against seven antifungals (anidulafungin, caspofungin, micafungin, fluconazole, itraconazole, posaconazole, and voriconazole) using CLSI methods. The study showed good activity of echinocandins and triazoles against Aspergillus spp. (MIC90/minimum effective concentration [MEC90] range, 0.015–2 μg/mL), but the echinocandins were not active against other molds (MEC90 range, 4 to >16 μg/mL).

In a 1-year study by Khan et al, a total of 110 isolates were tested against antifungal drugs amphotericin B, fluconazole, and voriconazole (CLSI M38-A2) by BMD method. The isolates were Aspergillus spp. (n = 45), Alternaria spp. (n = 40), and Cladosporium spp. (n = 25). Amphotericin B was found to be susceptible (MIC ≤ 1 μg/mL) in 37 (82.2%) patients with A. fumigatus, 35 (87.5%) patients with Alternaria alternata, and 19 (76%) patients with Cladosporium sphaerospermum isolate detected in clinical specimen. Amphotericin B, being the most effective drug, was resistant (MIC ≥ 4 μg/mL) in 13.4% (6) of patients with A. fumigatus, 2.5% (1) of patients with A. alternata, and 8% (2) of patients with C. sphaerospermum isolate detected in clinical specimen. The authors also mentioned in their study that broad-spectrum triazole deriva- tives such as voriconazole and itraconazole have comparable efficacy to amphotericin B and none of the isolated A. fumigatus was susceptible to fluconazole. Though every effort has been taken to compile the outcome of various studies related to the given topic, research enthusiasts are still requested to go through the references as mentioned for exhaustive yet detailed literature.

Conclusion
The study focuses on the fact that clinicians should switch from empirical treatment to susceptible drugs as early as
possible to combat antifungal resistance and newer mutations that horrify us every single day with poor patient outcomes.

Conflict of Interest
None declared.

References
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