In Vitro Antifungal Drug Susceptibility Testing of Aspergillus Isolates from Clinical and Environmental Samples

ABSTRACT

Background: Antifungal drug susceptibility testing (ADST) is still not done routinely for detection of drug resistance pattern of Aspergillus species. Physicians prescribe treatment to patients empirically which has led to the emergence of drug resistance in Aspergillus species.

Aim: The study aims to standardize the facility for antifungal drug susceptibility testing of Aspergillus species.

Materials and methods: In vitro drug sensitivity testing was done using the method as per Clinical and Laboratory Standards Institute (CLSI) guidelines.

Results: Drug sensitivity by phenotypic method revealed resistance to fluconazole for all Aspergillus species whereas miconazole, nystatin, and clotrimazole showed 100% sensitivity to all Aspergillus species. However, itraconazole and amphotericin - B showed 33.33–66.67% sensitivity, and ketoconazole showed 44.44–100% sensitivity.

Conclusion: Drug resistance testing by phenotypic method revealed that Aspergillus isolates from patient samples were resistant to fluconazole for all Aspergillus species whereas miconazole, nystatin, and clotrimazole showed 100% sensitivity to all Aspergillus species. Itraconazole shows 66.67–33.33% sensitivity. Amphotericin B shows 66.67–33.33% sensitivity. Ketoconazole shows 100–44.44% sensitivity to various Aspergillus species.

Keywords: Antifungal drugs, Aspergillus, Drug resistance, Disk diffusion method, E-test.

How to cite this article: Raksha, Singh G, Urhekar AD. In Vitro Antifungal Drug Susceptibility Testing of Aspergillus Isolates from Clinical and Environmental Samples. MGM J Med Sci 2018;5(4):154-158.

Source of support: Nil

Conflict of interest: None

INTRODUCTION

Fungal infection is a growing problem in the developed world. Fungi readily infect immunocompromised patients, and systemic infections typically cause high morbidity. Reports of fungal infections in healthy populations are also rising for example, because of the increased virulence of pathogens such as Aspergillus fumigatus. Fungi are now as a serious threat to human health as bacteria, viruses, and parasites. Aspergillus species cause a wide range of diseases including chronic, acute and sub-acute diseases. Invasive aspergillosis (IA) causes approximately 30% of fungal infections in patients dying with cancer. The crude mortality from IA is approximately 85% and falls to approximately 50% if treated. The most common isolates from clinical samples are Aspergillus fumigatus; other species like A. flavus, A. niger, and A. terreus may also cause infections.†

The gold standard antifungal drug is amphotericin B, while fluconazole and itraconazole are also used. Voriconazole may be useful against yeasts and filamentous fungi. The CLSI has developed broth microdilution method for antifungal drug sensitivity pattern of molds. Also, an agar diffusion method has been developed for yeasts by disk diffusion (CLSI M44-A). Still, no guidelines are available for antifungal drug susceptibility (ADS) of the mold by the disk diffusion method.† The aim of the present study aims to standardize a cheap and easy to perform a method to test the ADS of Aspergillus species.

MATERIALS AND METHODS

This prospective study was conducted at Mycology Laboratory, Department of Microbiology, Mahatma Gandhi Mission Medical College and Hospital, Kamalthe, Navi Mumbai, India, over a period of one year from January 2015 to December 2015. In vitro drug sensitivity testing was done using the method as per CLSI guidelines. For Disk diffusion testing of antifungal drugs, i.e., amphotericin-B, fluconazole, clotrimazole, itraconazole, ketoconazole, miconazole and nystatin (Fig. 1) and for E-test strip amphotericin- B, fluconazole, voriconazole, itraconazole and ketoconazole (Fig. 2) were purchased from HiMedia Company.
Disk Diffusion Method

Preparation of Inoculum
Test inoculum was prepared by adding mold (hyphae and spores) overnight old culture which was grown on Sabouraud’s dextrose agar after incubation at 37°C. Fungal growth was mixed with sterile saline in a test tube. Tubes were vortex on a vortex mixture and turbidity was adjusted equal to the 0.5 McFarland standard.

Test Procedure
Muller Hinton agar containing 2% glucose and 0.5 mg/mL methylene blue dye was prepared in Petri dish for antifungal drug susceptibility test.

A sterile cotton swab was moistened with prepared inoculums and dipped swab rotated on upper inside wall of the inoculums tube to avoid excess quantity of the inoculums. Inoculated onto Muller Hinton agar prepared plate by lawn culture method with the moistened swab three times. Inoculated plate cover with a lid and allowed to dry for 5–10 minutes.

Antifungal Disks were applied with the help of sterile pointed forceps under strict aseptic condition. Plates were incubated at 37°C in bacteriological incubator and 25°C in biological oxygen demand (BOD) incubator for 24 hours. After the incubation period of 24 hours, each plate was examined.

E-test Method

Preparation of Inoculum
Test inoculum was prepared by adding mold (hyphae and spores) overnight old culture which was grown on Sabouraud dextrose agar after incubation at 37°C. Fungal growth was mixed with sterile saline in a test tube. Tubes were vortex on a vortex mixture, and turbidity was adjusted equal to the 0.5 McFarland standard.

Test Procedure
Muller Hinton agar containing 2% glucose and 0.5 mg/mL Methylene blue dye was prepared in Petri dish for E-test. A sterile cotton swab was moistened with the and dipped swab rotated on upper inside wall of the tube to avoid excess quantity of the inoculums. Inoculated onto Muller Hinton agar prepared plate by lawn culture method with the moistened swab three times. Inoculated plate cover with a lid and allowed to dry for 5–10 minutes.

Kept Ezy MIC™ strip at room temperature for 15–20 minutes before applying. Ezy MIC™ strip was placed over the inoculated plate and within 1 minute Ezy MIC™ strip was absorbed and adhere with the inoculated plate surface. Plates were incubated at 37°C in bacteriological incubator and 25°C in BOD incubator for 24 hours. After the incubation period of 24 hours, each plate was examined.

Minimum Inhibitory Concentrations Reading
Plates were read-only when sufficient growth was seen. Minimum inhibitory concentrations (MIC) was read where the zone of inhibition may interfere with the MIC scale on the strip. After an overnight incubation period each plate was examined.

RESULTS
Aspergillus isolates from patient samples showed following drug susceptibilities: Fluconazole showed resistance for all Aspergillus species, miconazole, nystatin, and clotrimazole showed 100% sensitivity for all Aspergillus species.
species, itraconazole showed 66.67–3.33% sensitivity, amphotericin-B showed 66.67–33.33% sensitivity, and ketoconazole showed 100–44.44% sensitivity (Table 1).

Aspergillus isolates from environment showed following drug susceptibilities: Fluconazole showed resistance for all Aspergillus species, miconazole, nystatin, and clotrimazole showed 100% sensitivity to for Aspergillus species, itraconazole showed 100–40% sensitivity, amphotericin-B showed 100–40% sensitivity, and ketoconazole showed 55.56–33.33% sensitivity (Table 1).

Antifungal drug sensitivity was performed for amphotericin-B, fluconazole, itraconazole, ketoconazole, miconazole, nystatin, clotrimazole by disk diffusion method and also antifungal drug sensitivity was performed by E-test for amphotericin-B, fluconazole, itraconazole, ketoconazole, and voriconazole. Fluconazole showed resistance to all Aspergillus species from patient and environment (Table 2).

Disk diffusion test for antifungal drugs showed that sensitivity in patient samples was less than from environment. It means that aspergilli have developed resistance upon entry in patient’s tissues.

**DISCUSSION**

*In vitro* drug sensitivity was performed as per standard procedures mentioned in materials and methods. Drug sensitivity of aspergilli isolated from patient samples and the environment was studied and compared. Aspergillus isolates from patient samples showed the following findings: Fluconazole showed resistance for all Aspergillus species, miconazole, nystatin, and clotrimazole showed 100% sensitivity for all *Aspergillus* species, itraconazole showed 66.67–33.33% sensitivity, amphotericin-B showed 66.67–33.33% sensitivity, and ketoconazole showed 100–44.44% sensitivity. Aspergillus isolates from the environment showed the following findings: Fluconazole showed resistance for all *Aspergillus* species, miconazole, nystatin, and clotrimazole showed 100% sensitivity to for *Aspergillus* species, Itraconazole showed 100–40% sensitivity, amphotericin-B showed 100–40% sensitivity, and Ketoconazole showed 55.56–33.33% sensitivity (Tables 1 and 2).

Gupta et al. studied 44 isolates. Their results showed by disk diffusion method amphotericin B showed 87.5%, voriconazole (93.8%) and caspofungin (100%) and E-test amphotericin B 93.8%, voriconazole (93.8%) and caspofungin (100%). Espinel–Ingroff et al. studied on 555 isolates. Amphotericin B and itraconazole, voriconazole, caspofungin posaconazole. Disk diffusion test showed a satisfactory zone of inhibition in comparison of MICs, i.e., 91–100% versus 82–100% for 4 out of 5. Arikan, et al. studied on 78 isolates caspofungin. Caspofungin drug showed effective against *Aspergillus* species.

Serrano et al. studied 77 isolates. Disk diffusion method was found equally good as the results showed by E-test. Kazemi et al. studied on 50 isolates using Voriconazole, itraconazole and amphotericin B. Voriconazole was more sensitive than itraconazole with MICs between 0.5–1 µg/mL. It appears that all *Aspergillus* species from patient’s samples and environment have developed

**Table 1: Antifungal drug resistance pattern of *Aspergillus* species isolated from patient and environmental samples by disk diffusion test**

<table>
<thead>
<tr>
<th>Patient samples</th>
<th>FLC</th>
<th>IT</th>
<th>AP</th>
<th>KT</th>
<th>MIC</th>
<th>NS</th>
<th>CC</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. niger</em> (n = 18)</td>
<td>(0%)</td>
<td>(50%)</td>
<td>(65.56%)</td>
<td>(44.44%)</td>
<td>(100%)</td>
<td>(100%)</td>
<td>(100%)</td>
</tr>
<tr>
<td><em>A. fumigatus</em> (n = 9)</td>
<td>(0%)</td>
<td>(66.67%)</td>
<td>(66.67%)</td>
<td>(33.33%)</td>
<td>(100%)</td>
<td>(100%)</td>
<td>(100%)</td>
</tr>
<tr>
<td><em>A. flavus</em> (n = 7)</td>
<td>(0%)</td>
<td>(57.14%)</td>
<td>(42.86%)</td>
<td>(42.86%)</td>
<td>(100%)</td>
<td>(100%)</td>
<td>(100%)</td>
</tr>
<tr>
<td><em>A. brasiliensis</em> (n = 3)</td>
<td>(0%)</td>
<td>(33.33%)</td>
<td>(33.33%)</td>
<td>(100%)</td>
<td>(100%)</td>
<td>(100%)</td>
<td>(100%)</td>
</tr>
<tr>
<td><em>A. terreus</em> (n = 2)</td>
<td>(0%)</td>
<td>(50%)</td>
<td>(50%)</td>
<td>(100%)</td>
<td>(100%)</td>
<td>(100%)</td>
<td>(100%)</td>
</tr>
<tr>
<td>Environmental Samples</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. niger</em> (n = 20)</td>
<td>(0%)</td>
<td>(70%)</td>
<td>(70%)</td>
<td>(50%)</td>
<td>(100%)</td>
<td>(100%)</td>
<td>(100%)</td>
</tr>
<tr>
<td><em>A. fumigatus</em> (n = 9)</td>
<td>(0%)</td>
<td>(100%)</td>
<td>(88.89%)</td>
<td>(55.56%)</td>
<td>(100%)</td>
<td>(100%)</td>
<td>(90%)</td>
</tr>
<tr>
<td><em>A. flavus</em> (n = 5)</td>
<td>(0%)</td>
<td>(40%)</td>
<td>(40%)</td>
<td>(40%)</td>
<td>(100%)</td>
<td>(100%)</td>
<td>(100%)</td>
</tr>
<tr>
<td><em>A. brasiliensis</em> (n = 3)</td>
<td>(0%)</td>
<td>(100%)</td>
<td>(100%)</td>
<td>(33.33%)</td>
<td>(100%)</td>
<td>(100%)</td>
<td>(100%)</td>
</tr>
<tr>
<td><em>A. terreus</em> (n = 2)</td>
<td>(0%)</td>
<td>(100%)</td>
<td>(100%)</td>
<td>(50%)</td>
<td>(100%)</td>
<td>(100%)</td>
<td>(100%)</td>
</tr>
</tbody>
</table>

Abbreviations: FLC = Fluconazole, IT = Itraconazole, AP = Amphotericin-B, KT = Ketoconazole, MIC = Miconazole, NS = Nystatin, CC = Clotrimazole
resistance to fluconazole. Aspergilli from both groups show sensitivity to miconazole, nystatin, and clotrimazole. However, patient samples show less sensitivity to itraconazole, amphotericin-B, ketoconazole than the environment, suggesting the development of resistance because of certain conditions in patients.

In E-test, however, the sensitivity of Aspergillus isolates from patient and environment is similar in E-test measuring MIC. However, it is different in disk diffusion test. E-test measures the minimum inhibitory concentrations values of the organism, while the disk diffusion method measures the sensitivity to fixed, clinically required optimal concentration of the drug.

It is possible that the disk diffusion test is showing resistance at a particular concentration (e.g., 1 μg/mL) but E-test may show sensitivity at 1.5 μg/mL or 2 μg/mL. In this situation, if the patient needs a particular drug, it can be given in higher concentration but considering the side effects.

Antifungal drug sensitivity was performed for amphotericin-B, fluconazole, itraconazole, ketoconazole, miconazole, nystatin, clotrimazole by disk diffusion method and against amphotericin-B, fluconazole, itraconazole, ketoconazole, and voriconazole. Fluconazole showed resistance to all Aspergillus species from patient and environment.

Disk diffusion test for antifungal drugs showed that sensitivity in patient samples was less than from environment. It means that aspergilli have developed resistance upon entry in patient’s tissues. This resistance can be developed by organisms to counteract the antimicrobial agents used by patients.

Cost-effectiveness of any test is important to make it available to a greater number of suspect cases. At the same time, it is necessary to maintain quality control in relation to control strains, i.e., American type culture collection (ATCC) strains.

**CONCLUSION**

Comparison of sensitivity of Aspergillus species to antifungal drugs showed less sensitivity in patient isolates as compared to those isolated from the environment. It means that aspergilli in the environment have good sensitivity, but upon entry in patients, the aspergilli acquire greater resistance possibly because of struggle to survive in conditions which are directed to the elimination of aspergilli from patient tissues. The differences in sensitivity are observed in disk diffusion test where optimal drug concentration is used. However, there is no difference in sensitivity by E-test method which measures MIC. Minimum inhibitory concentration is a predetermined level in aspergilli which possibly cannot be modified in adverse conditions. Disk diffusion test is clearly cheaper than E-test. Disk diffusion test can be routinely performed while E-test can be performed whenever needed.

**REFERENCES**


