Original Article

Pattern of Antifungal Susceptibility in Pathogenic Molds by Microdilution Method at a Tertiary Care Hospital

Maria Khan*, Aamer Ikram, Gohar Zaman, Adeel Gardezi and Farida Khurram Lalani

Department of Microbiology, Armed Forces Institute of Pathology, National University of Management Sciences, CMH Rawalpindi, Pakistan

Corresponding author:
Maria Khan,
Department of Microbiology, Armed Forces Institute of Pathology, National University of Management Sciences, CMH Rawalpindi, Pakistan,
Tel: 0092-33191119 86;
E-mail: kmaria22@hotmail.com

Abstract

Fungal infections are increasingly being recognized especially in immunocompromised patients. Susceptibility of molds varies from species to species and timely generated reports can aid clinicians in the appropriate therapeutic decision. The study was conducted to determine the pattern of pathogenic molds in our tertiary care set-ups and the existing susceptibility by the standard broth microdilution method. The study was conducted in Armed Forces Institute of Pathology, Rawalpindi from January through December, 2016. Isolated fungal molds were tested against antifungal drugs by broth microdilution method; amphotericin B, fluconazole and voriconazole. A total of 110 isolates were tested by broth dilution antifungal susceptibility testing of filamentous fungi approved by the Clinical and Laboratory Standards Institute M38-A2 document. Mean age was 43 years and gender 79 (71.8%) patients were male and 31 (28.2%) patients were female. A total of 110 isolates (*Aspergillus* spp., n=45; *Alternaria* spp., n=40; and *Cladosporium* spp. n=25) were tested by broth dilution antifungal susceptibility testing of filamentous fungi. Amphotericin B was susceptible (MIC ≤1 µg/ml) in 37 (82.2%) of patients with *Aspergillus fumigatus*, 35 (87.5%) of patients with *Alternaria alternata* and (19) 76% of patients with *Cladosporium sphaerospermum*. While Amphotericin B was resistant (MIC ≥ 4 µg/ml) in (6) 13.4% of patients with *Aspergillus fumigatus*, (1) 2.5% in patient with *Alternaria alternata* and (2) 8% of in patients with *Cladosporium sphaerospermum*.

Susceptibility of mold in Fluconazole (MIC ≤ 8 µg/ml) was analyzed in which none of patients with *Aspergillus fumigatus* were sensitive, (1) 2.5% of patient with *Alternaria alternata* and none of patients with *Cladosporium sphaerospermum*. While Fluconazole was resistant (MIC ≥64 µg/ml) in (43) 95.6% of patients with *Aspergillus fumigatus*, (35) 87.5% of patients with *alternaria* and (21) 84% of patients with *Cladosporium sphaerospermum*.

Voriconazole was susceptible (MIC ≤0.125 µg/ml) in (38) 84.5% of patients with *Aspergillus fumigatus*, (33) 82.5% of patients with *Alternaria alternata* and (21) 84% of patients with *Cladosporium sphaerospermum*.

The isolated molds exhibited variable susceptibilities to antifungals. The increasing number of invasive mold infections, coupled with documented resistance to antifungal agents, has potentiated the need for having standardized methods for determining the *in vitro* susceptibilities of both new and established antifungal agents against clinical isolates of filamentous fungi.©

Keywords: Antifungal; Minimum Inhibitory Concentration (MIC); Amphotericin B; Fluconazole; Voriconazole

Introduction

The incidence of mold infection in both, the healthy and the immunocompromised, is steadily increasing. [1] These infections have become a health nuisance; mold infections in patients with malignancies continues to increase despite the widespread use of air filtration systems, suggesting the presence of other hospital sources for these molds. [2] Some commonly encountered pathogenic molds include *Cladosporium* species, *Aspergillus* spp, *Penicillium* spp and *Alternaria alternata*. *Cladosporium* has been known to cause several different types of infections, including skin, eye, sinus, and brain infections. [3] It is interesting that *Cladosporium sphaerospermum* can be isolated from the indoor air environment of asthmatics and non-asthmatics, which was confirmed in 26.3% of the cases investigated. [4]

The most comprehensive multicenter epidemiologic surveillance study of invasive fungal infections in transplantation has been from the Transplant-Associated Infection Surveillance Network (TRANSNET) database. [5] According to a multicenter survey in Italy done in 2013, the overall incidence of fungal infection was 16.5 cases per 1,000 admissions. 12.4% of these infections were due to molds. Among these, *Aspergillus fumigatus* was identified to be the most common pathogen. [6] The genus *Alternaria* cause is also responsible for causing a variety of opportunistic

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infections in humans. Cutaneous and subcutaneous infections are the most common (74.3%). Oculomycosis, invasive and non-invasive rhinosinusitis and onychomycosis have also been reported. [7] Broth dilution is the reference method for detection of MICs and antimicrobial susceptibility testing of antifungal agents. [8]

Such infections become a health nuisance especially in the immunocompromised patients despite spending tons of resources on prevention strategies such as use of air filtration systems or barrier precautions and even treatment. Isolation of fungal etiological agent is another challenge faced by the diagnostic labs of developing countries. Apart from yeasts commonly seen in superficial infections, molds have also been responsible for a wide variety of fungal infections encountered in clinical practice. Molds like *Aspergillus fumigatus*, *Alternaria alternata* and *Cladosporium sphaerospermum* contribute significantly to the spectrum of mycological infections in the clinical practice. Fungal isolates obtained from various clinical specimens and their susceptibility testing is warranted only when a true pathogen or otherwise clinically correlated isolate is grown from the sample. This is further complicated with limited availability of antifungal drugs susceptibility testing. Antifungal treatment without valid drug susceptibility results, keeps the patients and clinicians skeptical about the outcome.

Determining the resistance pattern of different molds to antifungals would enable us to effectively manage and treat patients. Broth microdilution antifungal susceptibility testing remains the standard for susceptibility testing that requires expertise as well as resources. Pakistan is a resource poor country where diagnosis and susceptibility testing for fungal infections have remained in the background. The studies on the subject are limited to establish spectrum of such infections in general or special patient population of this region and information on antifungal susceptibility testing is scarce. We carried out broth microdilution antifungal susceptibility testing of three major filamentous fungi namely *Aspergillus fumigatus*, *Alternaria* alternate and *Cladosporium sphaerospermum* isolated from clinical specimens. Our novel effort in Pakistan was directed to highlight epidemiological footprints of such isolates in our setup as well as to assist in therapeutic decision-making using standardized susceptibility testing method.

Material and Methods

The study was conducted from January to December 2016, at Department of Microbiology, Armed Forces Institute of Pathology, Rawalpindi, Pakistan. Various clinical samples including sputum, bronchoalveolar lavage, tissue and pus collected from both outdoor and admitted patients were dealt for the study. Specimens were subjected to microscopic examination by wet film preparation using lactophenol blue staining. The smears were observed under high power (40 X) objective lens. Presence of fungal hyphae in the wet film or otherwise was documented. Patients' demographic information with relevant clinical details was also recorded.

The specimens were inoculated at three points on each culture plate of a set of culture media comprising of Sabouraud's Dextrose agar (Oxoid™ Ltd CM 0041), Sabouraud's Dextrose agar with Chloramphenicol (Oxoid™ Ltd PO 0161), Sabouraud's Dextrose agar with Chloramphenicol and Actidione (Oxoid™ Ltd PO 0162). Each set of three plates was put in a dedicated zip locked plastic bag to prevent environmental or cross contamination from other specimens and incubated at 22ºC for 28 days. The plates were taken out of the incubation and the packing for aeration and observation for macroscopic growth on alternate days. Identical growth obtained on at least three inoculation points on minimum two out of three plates was taken as significant. The growth was identified using macroscopic colony morphology as well as microscopic features in Lactophenol blue preparation using a microscope. Plates showing, mixed growth of molds was excluded out of the study considering these as contamination. Once after final identification, molds were subjected to standardized antifungal susceptibility testing according to Clinical Laboratories Standards Institute (M38-A2). The selected isolates were further inoculated on Potato Dextrose Agar slant (Oxoid™ CM0139) for 5 to 7 days at 35°C. Colonies of molds exhibiting sporulation were covered by 1-2 ml of sterile 0.85% saline. The suspension was slightly agitated to mix the culture contents in it. Then the suspension is pipetted out over the slant, transferred to a sterile aliquot and then vortexed for 10-15 seconds. Optical densities (OD) of the suspensions were adjusted using spectrophotometer (530 nm) to 0.1 for *Aspergillus fumigatus*, 0.16 for *Cladosporium sphaerospermum* and 0.27 for *Alternaria alternata*. For broth microdilution, sterile 96 well microtiter disposable plates were used. For antifungal drug susceptibility testing, each well was inoculated with 0.1 ml of the inoculum suspension. Antifungal reference powders were obtained commercially (Sigma-Aldrich, Merck USA), from the drug manufacturer. The powders were stored at −20°C as recommended by the manufacturers. Antifungal stock solutions were prepared with concentration of 1280 µg/mL (10 times the highest concentration tested). Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich, Merck USA) was used as solvent for amphotericin B and voriconazole whereas water was for fluconazole. Sterile stock solutions were dispensed into sterile polypropylene vials.RPMI-1640 (Sigma-Aldrich, Merck USA) with glutamine and phenol red as, and without bicarbonate was used for testing the filamentous fungi. The pH of the medium was maintained between 6.9 and 7.1 at room temperature.The growth control wells contained 0.1 ml of the corresponding diluted inoculum suspension and 0.1 ml of the drug diluent (2%) without antifungal agent. Microdilution trays were then incubated at 35 °C for 48 hours. Some of the isolates were observed for 72 hours. Only three drugs were tested against the selected mold species. These drugs were selected for their easy availability in the country and better confidence of the clinicians of our setup over these antifungal agents.

Drug concentration ranges were:

- Amphotericin B 0.0313 to 16 µg/ml
- Voriconazole 0.0313 to 16 µg/ml
- Fluconazole 0.125 to 64 µg/ml

Microdilution trays were then incubated at 35 °C for 48 hours. Some of the isolates were observed for 72 hours. Minimal Inhibitory Concentration (MIC) was taken as the lowest antifungal agent concentration that achieves complete inhibition of the growth in the inoculated well. The MIC criteria for the three antifungals are shown in Table 1.

Aspergillus flavus ATCC® 204304 and *Aspergillus fumigatus* ATCC® 204305 were used as quality control strains.

Results

Department of Microbiology, Armed Forces Institute of Pathology Rawalpindi received a total of 110 specimens for fungal culture and sensitivity from January to December 2016. Specimens included tissues 96 (44.23%), sputum 53 (24.42%), bronchoalveolar lavage 39 (17.97%) and pus 29 (13.36%). Mean age of the patients was 43 years with SD ±15.629. Age distribution among 110 patients was analyzed as 31 (28.2%) patients were in the range 20-30 years, 21 (19.1%) patients in 31-40 years, 19 (17.3%) patients were in 41-50 years, 18 (16.4%) patients were between 51-60 years and 21 (19.1%) patients were in age range of 61-70 years. Seventy nine (71.8%) patients were male and 31 (28.2%) patients were female. Fifty-seven specimens (26.26%) did not reveal any fungal growth. Positive growths selected for antifungal susceptibility testing were obtained form 110 specimens. Frequencies of molds subjected to antifungal susceptibility testing from the study isolates were 45 (40.9%) *Aspergillus fumigatus*, 40 (36.3%) *Alternaria alternata*, and 25 (22.7%) *Cladosporium sphaerospermum*. Antifungal susceptibility testing of these isolates was carried out against amphotericin-B, voriconazole and fluconazole (Table 2).

Thirty seven (82.2%) *Aspergillus fumigatus* isolates were susceptible to amphotericin B and so as 35 (87.5%) *Alternaria alternata* and 19 (76%) *Cladosporium sphaerospermum*. Whereas amphotericin B was intermediate in 2 (4.4%) isolates of *Aspergillus fumigatus*, 4 (10%) isolates of *Alternaria alternata* and 4 (16%) isolates of *Cladosporium sphaerospermum*.

Voriconazole was found sensitive against 38 (84.5%) *Aspergillus fumigatus* isolates, 33 (82.5%) isolates of *Alternaria alternata* and 21 (84%) isolates of *Cladosporium sphaerospermum*. Susceptible Dose Dependent was observed in 6 (13.3%) *Aspergillus fumigatus* isolates, 6 (15%) isolates of *Alternaria alternata* and 2 (8%) isolates of *Cladosporium sphaerospermum*. Whereas voriconazole was resistant in 1 (2.5%) *Aspergillus fumigatus* isolate 1 (2.5%) isolate of *Alternaria alternata* and 2 (8%) isolates of *Cladosporium sphaerospermum*.

None of the *Aspergillus fumigatus* and *Cladosporium sphaerospermum* isolates were sensitive to fluconazole, while only 1 (2.5%) isolate of *Alternaria alternata* showed susceptibility to this antifungal agent. Susceptible Dose Dependent was observed in 2 (4.4%) of *Aspergillus fumigatus*, 4 (10%) of patients with *Alternaria alternata* and 4 (16%) of patients with *Cladosporium sphaerospermum*.

Discussion

With the availability of an increasing array of systemic antifungal agents, there is a need for accurate, reproducible and predictive susceptibility testing of fungal isolates in order to help inform clinical choice. The application of this standardized approach for the testing of yeast and mold isolates produces susceptibility results that are comparable between laboratories and allow epidemiological analyses at the national and even international level. In addition, monitoring and prediction of emerging susceptibility trends are now possible. Despite availability of many antifungal agents, antifungal clinical resistance occurs, perhaps as a consequence of an infecting organism found to be resistant *in vitro* to one or more antifungals tested. Thus, AFST results, if timely generated by the clinical microbiology laboratory and communicated to clinicians, can aid them in the therapeutic decision making, especially for difficult-to-treat invasive candidiasis and aspergillosis.

Although there are a variety of anti-fungal agents available, clinical resistance still occurs. This may be because of an infecting organism that is resistant *in vitro* or *in vivo*. With the incidence of fungal infections with high mortality rates on the rise, especially in the immune compromised, it is imperative that research be conducted regarding susceptibility testing to aid clinicians in therapeutic decision making. [9] Other susceptible patients are solid organ transplant recipients, those receiving

Table 1: MIC Criteria.

	Fluconazole	Amphotericin B	Voriconazole
Susceptible	≤8 µg/ml	≤ 1 µg/ml	≤ 0.125 µg/ml
Susceptible Dose dependent/ Intermediate	16-32 µg/ml	2 µg/ml	0.25-0.5 µg/ml
Resistant	≥ 64 µg/ml	≥ 4 µg/ml	≥ 1 µg/ml

Table 2: Susceptibility pattern of molds to anti-fungal agents.

Molds	Sensitivity results	Antifungal (%)		
		Amphotericin B	Fluconazole	Voriconazole
Aspergillus fumigatus (n=45)	S	82.2	0	84.5
	I	4.4	4.4	13.3
	R	13.4	95.6	2.2
Alternaria alternata (n=40)	S	87.5	2.5	82.5
	I	10	10	15
	R	2.5	87.5	2.5
Cladosporium sphaerospermum (n=25)	S	76	0	84
	I	16	16	8
	R	8	84	8

high doses of corticosteroids, patients in intensive care units and those with other immunodeficiencies. The risk of invasive fungal disease (IFD) in patients with malignancies depends on the underlying neoplastic process and on the degree of immunosuppression. [10]

Mycotic infections do not rank with bacterial, viral, helminthic and protozoal infections as causes of human suffering. However, they may still be a cause of major distress or disability in affected individuals. Also, in the past two decades, major advances in health care management have led to an increase in life threatening infections caused by true pathogenic or opportunistic fungi. For many years, only a few species of fungi (yeasts, molds and dimorphic fungi) were known to cause human infections. But an increase in size of the population at risk, along with the ubiquitous presence of fungi, has led to a great expansion in the fungal etiology. Fungal infections are often insidious and their diagnosis is often delayed due to co-existing illnesses. The emergence of these infections has created a challenge in their diagnosis and management. [11]

Our study shows almost one third of the patients from younger population with mean age 43 years with male predominance (Male: Female = 72: 28). Amphotericin B still appeared to be the most effective antifungal agent showing higher degree of susceptibility in *Aspergillus fumigatus* and *Alternaria alternata* (82.2% and 87.5% respectively) and slightly lesser degree in *Cladosporium sphaerospermum* (76%). According to a study conducted in the UK, out of 519 isolates of *A. fumigatus*, frequency of itraconazole was 5%. This number has siginificantly increased since 2004. [12] Another study conducted in India on ocular isolates, concluded that amphotericin B was susceptible in all Candida spp. Of the filamentous fungi tested, 4.6% were resistant to amphotericin B, 7.6% and 37.7% were resistant to ketoconazole and fluconazole respectively. [13] Another multicenter retrospective analysis of 83 cases showed, that the most frequently isolated genus was *Aspergillus* terreus isolated in 55% of cases while in remaining *A. terreus* was isolated along with other fungal pathogens. The most common pathogen isolated with *A. terreus* was *A. fumigatus* (48% of co-occurring isolates), followed by *A. flavus* (15%), *A. versicolor* (7.5%), and *A. nidulans* (5%). Patients mortality in the group treated with voriconazole was 55.8%, and was 73.4% in the group that received other antifungal therapy. [14]

Mycology Reference Centre Manchester, United Kingdom had reported azole resistance in *Aspergillus fumigatus* isolates, which was noted as a progressive increase in patients with Azole-resistant strains from 5% in 2004 to 20% in 2009. [8] In our study, voriconazole resistance (MIC ≥ 1µg/ml) was observed in 2.2% of patient with *aspergillus*, 2.5% of patient with *alternaria* and 8% patients with *cladosporium*. Another study showed that Ravuconazole and voriconazole were the most active agents tested against C. krusei (MIC90 of 0.5 µg/ml). Among *Aspergillus* spp., *A. fumigatus* was the most commonly (71.2% of isolates) recovered species; 96.2, 96.2, 84.6, and 11.5% of strains were inhibited by ≤1 µg/ml of ravuconazole, voriconazole, itraconazole, and amphotericin B, respectively. Of the antifungal agents tested, ravuconazole and voriconazole displayed the greatest spectrum of activity against pathogenic Candida and *Aspergillus* spp., regardless of geographic origin. These results extend upon previous findings from SENTRY Program reports (1997 to 2000). [15]

In this study amphotericin B resistant (MIC ≥ 4 µg/ml) isolates were 13.4% of patients with *Aspergillus fumigatus*, 2.5% in patient with *alternaria* and 8% of in patients with *cladosporium*. Although very little data are available regarding correlation between MIC and outcome of treatment with amphotericin B for the filamentous fungi, MICs above 2 µg/mL have been associated with treatment failures and MICs below 2 µg/mL with clinical cure among 29 patients treated with amphotericin B for invasive Aspergillosis caused by A. fumigatus (eight cases), *Aspergillus flavus* (12 cases), and A. terreus (nine cases). [16]

A report of azole resistance in *Aspergillus fumigatus* isolates submitted to the Mycology Reference Centre Manchester, United Kingdom, noted a progressive increase in patients with Azole-resistant strains from 5% in 2004 to 20% in 2009. [17] According to the SENTRY program, the prevalence of polyene resistance among *Aspergillus* species has increased remarkably, with only 11.5% of A. fumigatus isolates inhibited at ≤1 µg/mL. [15]

Conclusion

In conclusion, we are at an exciting stage in the development of *in vitro* antifungal susceptibility testing and systems are now available that allow the clinical laboratory to perform these tests with some confidence. Traditionally Amphotericin B has been the mainstay of antifungal treatment against invasive fungal infection. In our study, none of isolated *Aspergillus fumigatus* in our study was susceptible to fluconazole. On the contrary, broad-spectrum triazole derivatives like voriconazole, itraconazole etc. have demonstrated comparable efficacy to Amphotericin B. These results further potentiate the role of these antifungal agents in fungal infections making them an acceptable choice for empirical, prophylactic or targeted therapy in our clinical settings. This comparison requires to be further investigated at other set ups to be made a part of experts recommendation and hospital protocols.

Conflict of Interest

All authors disclose that there was no conflict of interest.

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