ORIGINAL ARTICLE

Comparison of the activities of amphotericin B, itraconazole, and voriconazole against clinical and environmental isolates of *Aspergillus* species

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ABSTRACT

Background: Invasive fungal infections are a significant cause of morbidity and mortality in immunocompromised populations. **Aims:** To evaluate the susceptibility pattern of our isolates against amphotericin B, itraconazole, and voriconazole and to compare the antifungal activities of these agents with each other against the *Aspergillus* species tested. **Settings and Design:** A prospective study was designed to include clinical and environmental isolates of *Aspergillus* species. **Materials and Methods:** 420 sputum samples, 70 bronchoalveolar lavage fluids, 160 oral washings, and 47 environmental samples were collected. Direct microscopy by potassium hydroxide and lactophenol cotton blue mounts followed by culture on Sabourad`s dextrose agar (SDA) was done. Susceptibility testing was performed by the broth microdilution technique as per Clinical Laboratory Standards Institute standards (M-38A). Additionally, all the isolates were also tested by the colorimetric microdilution technique using Alamar Blue dye. **Statistical Analysis:** It was done by the Chi-square test and Z-test using SPSS statistical software version 12.0. **Results and Conclusion:** Twenty-seven isolates (47.3%) were recovered from patients with chronic bronchial asthma followed by fibrocavitary pulmonary tuberculosis in 9 (15.7%), allergic bronchopulmonary aspergillosis (ABPA) in 6 cases (10.5%), bronchiectasis in 3 (5.2%), bronchogenic carcinoma in 5 (8.7%) and those receiving radiotherapy for head and neck cancer 7 (12.2%). Thirteen environmental isolates were also included in the study. The most common isolate was *A. fumigatus* 28 (40%), followed by *A. niger* 22 (31%), *A. flavus* 13 (19%), and *A. terreus* 7(10%). All isolates were susceptible to amphotericin B, itraconazole, and voriconazole. Among the three agents tested, voriconazole exhibited lowest MICs (≤1 μg/ml) against all *Aspergillus* species.

KEY WORDS: Amphotericin B, *Aspergillus*, Alamar Blue, broth microdilution, itraconazole, voriconazole

INTRODUCTION

The incidence of fungal infections has increased greatly over the past two decades especially in the immunocompromised host.[1] The important risk factors for these infections include prolonged neutropenia, chronic administration of corticosteroids, the widespread use of newer and more powerful cytotoxic and antibacterial drugs, and the increasing prevalence of HIV infection.[2] New fungal pathogens and fungi that were considered non-pathogenic have also emerged as etiologic agents of systemic disease.[3]

Paralleling the increasing incidence of invasive mycoses is the proliferation of newer antifungal agents. These are lipid formulations of amphotericin B, the newer triazoles such as voriconazole, ravuconazole, posaconazole, isavuconazole, and the echinocandin group of drugs.[4,5] In addition, an increasing number of reports suggest that a clinically significant resistance to conventional agents exists is some strains.[6]

Moreover, as with bacterial infections, the clinician would like to be guided by knowledge of local epidemiological patterns derived from drug susceptibility testing results. Unlike antibacterial susceptibility testing, however, antifungal sensitivity testing is largely in its infancy especially in developing nations. A prodigious array of techniques has been described, but without standardization, the various methods have produced widely discrepant results.[7-10]

The Clinical Laboratory Standards Institute (CLSI) has developed reference broth microdilution procedures for antifungal susceptibility testing of both yeasts and filamentous fungi published as documents M-38A.[11]

Among filamentous fungi, *Aspergillus* is

arguably the most important of the invasive mycoses. Antifungal susceptibility testing and treatment for these mycoses is still far from optimal.[12]

All these factors prompted the present study to evaluate the susceptibility pattern of our isolates against amphotericin B, itraconazole, and voriconazole and to compare the activities of these agents with each other against the *Aspergillus* species tested.

MATERIALS AND METHODS

A prospective study was conducted and relevant samples (sputum, bronchoalveolar lavage fluid, oral swab/washings) collected from patients attending the Chest and Tuberculosis Department, other medical outpatient (OPDs) and patients admitted in wards. All patients with clinically suspected Aspergillosis were included in the study. Sample processing was done in the same institute (Dept. of Microbiology).

Isolates

Seventy clinical and environmental isolates of *Aspergillus* spp. (*A. fumigatus* [n=28], *A. niger* [n=22], *A. flavus* [n=13], *A. terreus* [n=7]) were included in the study. Identification of these isolates was based on microscopy by potassium hydroxide (KOH) and lactophenol cotton blue (LPCB) mounts followed by culture on two tubes of Sabouraud dextrose agar (SDA), one at 25° C and the other at 37° C. Microslide culture was done where needed. Two quality control organisms *A. flavus*, (ATCC 204304) and *A. fumigatus* ATCC (204305) were included each time a set of isolates was tested with each drug. The control isolates were obtained from the Institute of Microbial Technologies (IMTech), Chandigarh.

Antifungal Agents

Amphotericin B (Sigma-Aldrich, Bangalore, India), itraconazole (Janssen, New Jersey, USA), and voriconazole (Pfizer, New York, USA) were kindly provided by their respective manufacturers as standard powders. All three drugs were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, Bangalore, India)) and stock solutions were stored at –70°C. The highest desired test concentration was 1600 μg/ml. Hence, 4.8 mg of the antifungal agent was weighed and dissolved in 3 ml of the solvent i.e. DMSO (assuming100% potency of the drug). A series of dilutions at 100 times the final concentration (100×) was prepared from the antifungal stock solution in the same solvent (i.e. DMSO). For the broth microdilution test the intermediate solution was diluted 1:50 to final strength in the test medium i.e. RPMI 1640 buffered with 0.165 mol/l MOPS,3-(N-Morpholino) propanesulfonic acid (Hi-Media, Mumbai, India).This gave 2× the strength of the working solution. The dilution series were in the range 16 μg/ml to 0.0313 μg/ml for all the drugs.

Inoculum Preparation

The inoculum preparation was as per Clinical Lab Standards Institute (CLSI) document M38A. In brief, conidia formation was induced by growing the isolates on cornmeal agar at 35°C for 7 days. Seven day old colonies were covered with 1 ml of sterile normal saline and the conidia were harvested by probing the colonies with the tip of a sterile Pasteur pipette. The resulting mixture of conidia and hyphal particles was transferred to a sterile tube. Heavy particles of the suspension (when they were present) were allowed to settle for 3 to 5 min and the upper homogenous suspension after vortexing for 15 s was adjusted to optical densities that ranged from 0.09 to 0.11 (at 625 nm) i.e. 80% to 82% transmittance using a spectrophotometer (Hitachi, Japan). For the microdilution test, the inoculum suspension was diluted 1:50 in RPMI that corresponded to two times the density (2×) needed for the test of approximately 0.4×10^4 to 5×10^4 CFU/ml. The test inoculum was made in sufficient volume to directly inoculate each tube with 0.1 ml of the corresponding diluted inoculum suspension. A growth control well containing RPMI-1640 broth without antifungal agents was put each time a set of isolates were tested. Inoculum quantification for each isolate was performed by plating 0.01 ml of a 1:100 dilution of the adjusted inoculum suspension on SDA plates to determine the viable number of colony forming units per milliliter. The plates were incubated at 37°C and observed daily for fungal colonies.

Broth Microdilution Test

Each microdilution well was inoculated with 100 μl of the diluted 2× drug concentration and 100 μl of the diluted 2× conidial inoculum suspension. The final volume in each well was 200 μl. The microplates were incubated at 35 °C without agitation and examined after 48 h for determining minimal inhibitory concentration (MIC) results.

Colorimetric Determination of MICs Using Alamar Blue Dye

Colorimetric determination of MICs was done by adding an oxidation- reduction indicator to each well at the time of inoculation, 25 μl of Alamar Blue (Serotec Ltd, U.K. C/o OSB Agencies, India) per well. The plates were then incubated at 35 °C and read at 48 h. Growth in each well was indicated by a color change from dark blue/purple to pink. The MIC was recorded at the first blue well.

Reading of Plates

For the non-colorimetric methods, the growth in each well was compared with that of the growth control with the aid of a reading mirror. The wells were then given a numerical score as follows: a score of 0 (zero) i.e. optically clear well for amphotericin B; a score of 2 (50% reduction in turbidity) for the azoles. The score of 2 was compared with a 1:5 dilution of the growth control well. For the colorimetric method the MIC was defined as the lowest concentration of the antifungal agent preventing the development of a red color (i.e. first blue well).

Statistical Analysis

This was done by Chi-square test and Z-test using SPSS statistical software version 12.0 (SPSS Inc., Chicago, IL, USA)

Table 1: *Aspergillus* species isolated from various clinical diseases

Disease	*Number of patients from whom samples were collected*	*Number of Aspergillus species isolated*	*% of Aspergillus species isolated*
Chronic bronchial asthma (on steroid therapy)	63	27	47.37
Fibrocavitary pulmonary tuberculosis	27	9	15.79
Allergic bronchopulmonary aspergillosis	17	6	10.53
Bronchiectasis	15	3	5.26
Bronchogenic carcinoma	10	5	8.77
Head-neck cancer	8	7	12.28
Total	140	57	41

RESULTS

In the present study, a maximum number of *Aspergillus* isolates 27 (47.3%) were recovered from patients with chronic bronchial asthma followed by fibrocavitary pulmonary tuberculosis 9 (15.7%), allergic bronchopulmonary aspergillosis (ABPA) cases 6 (10.5%), bronchiectasis 3 (5.2%), bronchogenic carcinoma 5 (8.7%), and those receiving radiotherapy for head and neck cancer 7 (12.2%) [Table 1]. Although sputum was the most common specimen collected (n=420), the highest number of isolates were from environmental samples (27.6%) followed by lavage fluid (24.29%) [Table 2]. Four different species of *Aspergillus* were isolated from various clinical conditions/specimens and the most common isolates were *A. fumigatus* 28 (40%), followed by *A. niger* 22 (31%), *A. flavus* 13 (19%), and *A. terreus* 7 (10%).

In the present study, all the isolates were screened for their geometric mean MIC and the MIC range at 48 h for three different drugs i.e. amphotericin B, itraconazole, and voriconazole. The highest MIC value observed for amphotericin B and itraconazole by the microdilution method was 4 μg/ml, whereas by the Alamar Blue method it was 8 μg/ml. The voriconazole MICs spanned a comparatively narrow range with the maximum value being 2 μg/ml. We observed that among the four species of *Aspergillus* tested, the MICs for *A. terreus* were the lowest (0.0313-2 μg/ml). It was also observed that the overall values obtained by the colorimetric method tended to be higher in comparison to the microdilution method.

Tables 3-5 show the geometric mean MIC (MIC± Standard Deviation) and the MIC range in μg/ml of amphotericin B, itraconazole, and voriconazole, respectively, at 48 h by both methods.

Table 3 shows the geometric mean MIC and MIC range (μg/ml) of amphotericin B at 48 h. The lowest MIC value for *A. fumigatus* was 0.5 μg/ml by the microdilution method, while the maximum value was 8 μg/ml by the Alamar Blue format. For *A. niger,* the minimum MIC value was 0.5 μg/ml by the microdilution format while the maximum value was 4 μg/ml by both the microdilution and colorimetric formats. For both *A. flavus* and *A. terreus* the minimum MIC value by the microdilution method was 0.0313 μg/ml.

Table 4 shows the geometric mean MIC and MIC range (μg/ml) of itraconazole at 48 h. For all the four species of *Aspergillus* the lowest MIC value by the microdilution method was 0.0313 μg/ml. The highest MIC value for *A. fumigatus, A. niger,* and *A. flavus* was 4 μg/ml by both the methods. The only exception was *A. fumigatus* for which the maximum MIC value was 8 μg/ml by the Alamar Blue method. For *A. terreus,* the MIC value was between 0.5 and 2 μg/ml.

Similarly, Table 5 shows the geometric mean MIC and MIC

Table 2: Total number of isolates in relation to various samples

Samples	*Total samples collected*	*Total number of isolates*	*%age of Aspergillus species isolated*
Sputum	420	32	7.6
BAL	70	17	24.29
Oral swab/oral washing	160	8	5.0
Environmental	47	13	27.6
Total	697	70	10.0

Table 3: Geometric mean MIC and MIC range (μg/ml) of amphotericin B at 48 h by the microdilution and Alamar Blue methods

Species	*Microdilution*	*Colorimetric*
A. fumigatus (n=28)	2.14 2 ± 1.31 (0.5-4)	2.222 ± 2.5 (1-8)
A. niger (n= 22)	1.38 2 ± 1.26 (0.5-4)	0.9 2 ± 1.09 (0.5-4)
A. flavus (n=13)	1.34 ± 1.02 (0.313-4)	1.96 ± 1.53 (0.5-8)
A. terreus (n= 7)	0.29 ± 0.16 (0.0313-0.5)	0.32 ± 0.18 (0.5-2)

Table 4: Geometric mean MIC and MIC range (μg/ml) of itraconazole at 48 h by the microdilution and Alamar Blue methods

Species	*Microdilution*	*Colorimetric*
A. fumigatus (n=28)	1.26 ± 1.13 (0.0313-4)	2.44 ± 2.30 (0.25-8)
A. niger (n= 22)	1.61 ± 1.31 (0.0313-4)	2.59 ± 1.39 (0.5-4)
A. flavus (n=13)	0.36 ± 0.15 (0.0313-4)	0.92 ± 0.64 (0.5-4)
A. terreus (n= 7)	0.36 ± 0.18 (0.0313-0.5)	0.92 ± 0.73 (0.5-2)

Table 5: Geometric mean MIC and MIC range (μg/ml) of voriconazole at 48 h by the microdilution and Alamar Blue methods

Species	*Microdilution*	*Colorimetric*
A. fumigatus (n=28)	0.21±.23 (0.0313-1)	0.49±.31 (0.0313-1)
A. niger (n= 22)	0.48±0.30(0.0313-1)	1.61± 1.42 (0.25-2)
A. flavus (n=13)	0.36±0.15 (0.0313-1)	0.92±0.64 (0.0313-1)
A. terreus (n= 7)	0.19±0.17 (0.0313-0.5)	0.43±0.17 (0.0313-0.5)

Table 6: MIC90 of the four *Aspergillus* species determined by microdilution and colorimetric methods. A, I, and V represent amphotericin B, itraconazole, and voriconazole, respectively. The values represent µg/ml

Method	*A. fumigatus*			*A. niger*			*A. flavus*			*A. terreus*		
	A	*I*	*V*	*A*	*I*	*V*	*A*	*I*	*V*	*A*	*I*	*V*
Microdilution	4.0	4.0	0.5	4.0	1.0	1.0	2.0	0.5	0.5	0.5	0.5	0.25
Colorimetric	4.0	2.0	1.0	4.0	4.0	1.0	4.0	2.0	1.0	2.0	2.0	0.5

range (µg/ml) of voriconazole at 48 h. For all the four species of *Aspergillus* the lowest MIC value was 0.0313 µg/ml by both the testing formats. The only exception was *A. niger* for which the minimum MIC value was 0.25 µg/ml by the Alamar Blue method. The maximum MIC value was between 0.25 and 2 µg/ml for all the species tested.

For all the four species of *Aspergillus* tested against amphotericin B, itraconazole, and voriconazole, a statistically significant difference was found with the azoles when the Alamar Blue method was compared to the microdilution method against itraconazole and voriconazole ($P < 0.05$). The MIC at which 90% of the isolates were inhibited for each of the four species is also lowest for voriconazole [Table 6]. Further, voriconazole is the only agent inhibiting 100% of the isolates at an MIC of ≤1 µg/ml.

DISCUSSION

The goal of this study was to evaluate the susceptibility pattern of our isolates against amphotericin B, itraconazole, and voriconazole and to compare the activities of these agents with each other against the *Aspergillus* species tested. We followed the standard procedure as recommended by CLSI which included buffered RPMI 1640 medium, a 37°C incubation temperature and a spectrophotometric method of inoculum preparation. The MIC readings were scored as 0, optically clear well for all the drugs since a previous study by Pfaller *et al*.[13] has indicated that this less stringent criterion is more reliable for end point determination. The usual partial inhibition observed with the azoles makes the determination of MIC endpoints critical. All the isolates in our study were susceptible to amphotericin B, itraconazole, and voriconazole (MIC ≤ 4 µg/ml). The MIC range and the geometric mean MIC were lowest for voriconazole. Among the four species of *Aspergillus* tested *A. terreus* was the most susceptible with the maximum MIC value of 2 µg/ml. This is in agreement with the works of other authors.[13-16]

In the colorimetric method using the Alamar Blue dye we found that 24 h of incubation was not sufficient for the complete conversion of Alamar Blue to its pink derivative. Clear end-points were obtained between 30 and 40 h of incubation. Before 40 h visible growth without a color change was also observed for some isolates. Meletiadis *et al*.[9] have reported similar findings.

The geometric mean MIC for all the three agents, amphotericin B, itraconazole, and voriconazole, against all four species of *Aspergillus* was high. This difference was not statistically significant for amphotericin B. It was however statistically significant in the case of itraconazole and voriconazole; P was <0.05 when the microdilution method was compared with the Alamar Blue method. Pfaller *et al*.[17] have also reported higher MICs with the Alamar Blue method when they tested fluconazole against 119 Candida isolates. In their study the high off-scale MICs were converted to the next highest concentration while the low off scale MICS were left unchanged.

One reason for the higher MICs by the colorimetric microdilution method could be that the dye will change color (pink) with small amounts of growth which may not be evident by the visual method of MIC determination. The usual partial inhibition observed with the azoles makes the determination of MIC endpoints critical. Another reason could be that readings were taken at 48 h and not at 24 h. MICs tend to be higher as the duration of incubation increases.

In our study, we have also tried to compare the activities of various antifungal agents with each other. Among amphotericin B, itraconazole, and voriconazole the geometric mean MIC value of amphotericin B was highest for all the species of *Aspergillus* by both the methods (i.e. broth microdilution and colorimetric microdilution) while that for voriconazole was the lowest. This is in agreement with the works of other authors.[18-20] In their series of strains tested, none had a MIC value of >4 µg/ml). The MIC at which 90% of the isolates were inhibited for each of the four species is also lowest for voriconazole [Table 6]. Further, voriconazole is the only agent inhibiting 100% of the isolates at an MIC of ≤1 µg/ml.

The results of this study confirm and extend the previous comparisons of amphotericin B, itraconazole, and voriconazole. Voriconazole is more active *in vitro* than amphotericin B or itraconazole against all the species of *Aspergillus* tested. The higher MICs with the colorimetric method are statistically significant in the case of azoles. However, the Alamar Blue format is simple, provides clear end-points for visual determination of MICs, and can be automated.

In conclusion, studies regarding the susceptibility pattern of opportunistic fungal pathogens from India are few. Correlation of *in vitro* results with that of *in vivo* data would give a better idea about the efficacy of the tested drugs.

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