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IN VITRO SUSCEPTIBILITY TESTING OF INDOOR FUNGI BY ETEST

ANIRBAN CHAUDHURI[1], SUBARNA BHATTACHARYYA*[1] AND PUNARBASU CHAUDHURI[2]

[1] School of Environmental Studies, Jadavpur University, Kolkata 700 032, W.B., India
[2] Department of Environmental Science, University of Calcutta, Kolkata, W.B., India

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Abstract – Antifungal susceptibility tests have become important tools to guide the treatment of invasive fungal infections and also to detect antifungal resistance. Susceptibilities to antifungal drugs were determined by Etest for 50 isolates from indoor environments. Growth inhibition ellipses were uniform and well-delineated, and the points of intersection with the Etest strips were clearly determined after 48 h of incubation at 27 °C. According to the obtained results, all strains were found to be resistant to Fluconazole, whereas *Aspergillus* sp. was most susceptible for Posaconazole and Itraconazole antifungal agent. 90% of isolates were susceptible to Posaconazole and Itraconazole. In the present study, Posaconazole and Itraconazole were the most effective drugs for all isolates. Determining the susceptibility pattern is mainly required to assist clinicians in treating most superficial dermatophyte infections more effectively.

INTRODUCTION

In 1960s when antibiotic therapies were developed, a drastic rise in fungal infections was observed, and they currently represent a global health threat. Fungal infection can be very serious and, especially for those with compromised immune systems, even life threatening. Most fungi are harmless to humans, but there are several that can cause harmful infections, especially in people living with human immunodeficiency virus (HIV). These include aspergillosis, candidosis, coccidioidomycosis, cryptococcosis, histoplasmosis, mycetomas, mucormycosis, and paracoccidioidomycosis. Candidiasis has become the most frequent fungal infection in patients with Aids/cancer. Infact, dermatophytic and keratinophilic fungi can attack eyes, nails, hair, and mostly skin and initiate local infections such as ringworm and athlete's foot. Due to the life threatening nature of these infections and reports of drug resistance, susceptibility testing of indoor fungi has become very important.

To battle present fungal infections, researchers at various universities are also testing vaccines, new anti-fungal medicines or combination of medicines in clinical trials. The rapid increases in fungal infections specify an increasing demand for fast and accurate methods for antifungal screening and susceptibility testing. Various methods such as both micro & macro dilution, agar dilution, Etest, colorimetric micro dilution and disk diffusion have been available (Karaca *et al.,* 2004*;* Pujol *et al.,* 2002). Agar-based methods are attractive due to their simplicity. Among the commercial alternatives, the Etest (previously known as Epsilometer test) for yeasts is a novel agar diffusion procedure which is based on the diffusion of a continuous concentration gradient of the antifungal agent tested from a plastic strip into an agar medium (Espinel-Ingrofe *et al.,* 1996). This methodology has been adapted to a number of anti-fungal drugs such as Noxafil (Posaconazole), Diflucan (Fluconazole), Sporanox (Itraconazole), Vfend (Voriconazole), or Nizoral (Ketoconazole) called "azoles" are commonly used to fight common fungal infections.

Azole antifungal agents are considered to be first-line therapeutic drugs for fungal infections because of their effective antifungal activity and good safety profile (Hashemi *et al.,* 2015*;* Nabili *et al.,* 2016). The first azole was synthesized in 1944 by Woolley (Woolley, 1944), but it was not until 1958 that scientific community began to consider azoles as potential antifungal agents. Azoles are by far the most commonly used antifungals in clinical practice, and consequently, they are also the mostly studied by the scientific community regarding their

mode of action, their pharmacological properties, and the resistance mechanisms developed by microorganisms (Vandeputte *et al.*, 2012).

In late 1960s, Clotrimazole, Econazole, and Miconazole became available for the treatment (Fromtling, 1988). In addition, the adverse health effects or toxicities of the available antifungal agents have also limited their use in clinical practice (Campoy *et al.*, 2017; Burgess *et al.*, 1972; Tettenborn, 1974). Thus, discovering new antifungal agents or overcoming drug resistance has become breaking news in the antifungal field. Therefore, a new and fast procedure using potato dextrose agar (PDA) as a culture medium was developed for susceptibility testing of antifungal drugs. To this respect, the objective of the current study was to determine the susceptibility pattern of airborne fungi found in different indoor environments against a panel of antifungal drugs by means of the Etest.

MATERIALS AND METHODS

Fungal isolates and antifungal agents

Fifty fungal species (Table 1) belonging to *Aspergillus* sp. (n = 12), *Aspergillus niger* (n = 15), *Aspergillus flavus* (n = 5), *Aspergillus ficuum* (n = 3), *Curvularia* sp. (n = 2), *Penicillium* sp. (n = 9), *Rhizopus* sp. (n =1), *Rhizopus stolonifer* (n = 2), *Trichoderma* sp. (n = 1) isolated from different indoor environments of Kolkata (library, classroom, teachers room, gymnasium and kitchen) were used to evaluate the minimum inhibitory concentration (µg/mL) of three antifungal agents namely Posaconazole (HIMEDIA EM120), Fluconazole (HIMEDIA MD072) and Itraconazole (HIMEDIA MD073) in laboratory condition. Also, these strains had been identified from Agharkar Research Institute, Pune, India. The tested concentration of Posaconazole, Fluconazole and Itraconazole ranged from 0.002 to 32 µg/mL, 0.016 to 256 µg/mL and 0.002 to 32 µg/mL respectively. The strips were stored at -20 °C until the day that the MICs were determined.

Spore suspension preparation

1.8×10^5 spores/mL of all fifty fungal samples were used for the fungal mat preparation. One loop of spore was suspended into sterile distilled water.

Media used and preparation of plate for antifungal assay

The potato dextrose agar (PDA) medium (HIMEDIA M096) was used for the isolation and maintenance of fungal strain and also assessing the MIC of three antifungal agents namely Posaconazole, Fluconazole and Itraconazole in laboratory condition. The spore suspensions of each fungus were sprayed on the PDA plate separately in aseptic conditions. Antifungal strips were picked with a sterile forceps and placed on agar plate swabbed with test culture. The plates were kept in the incubator at 27 °C for 48 h till the lawn growth of test fungi visible on the agar plate. Inhibition of fungal growth produces an ellipse and the concentration was read at the intersected point of the ellipse produced.

RESULTS

The plates were examined after 48 h of incubation because minimum inhibitory concentrations of antifungal agent were dependent on the time period of incubation and, to a certain extent, on the species or genus tested. The inhibition of growth was observed among the strains with micro and macro colonies within them (Fig. 1).

Table 1. Sources of different indoor fungi

Fungus	Sources				
	Library	Classroom	Teachers room	Gymnasium	Kitchen
Aspergillus sp. (12)				✓	✓
Aspergillus niger (15)	✓		✓	✓	✓
Aspergillus flavus (5)			✓		✓
Aspergillus ficuum (3)		✓	✓		
Curvularia sp. (2)	✓	✓			
Penicillium sp. (9)	✓		✓	✓	✓
Rhizopus sp. (1)				✓	
Rhizopus stolonifer (2)					✓
Trichoderma sp. (1)	✓				

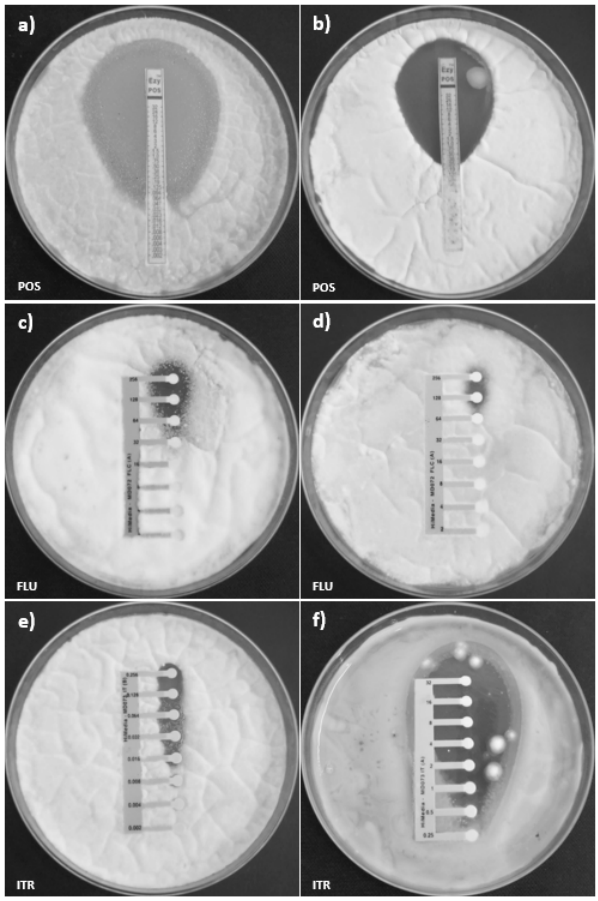

Fig. 1. Zone of inhibition a) *Aspergillus niger*, ellipse with microcolonies, MIC: 0.023 µg/ml b) *Penicillium* sp., ellipse with a macrocolony, MIC: 0.25 µg/ml c) *Penicillium* sp., ellipse with microcolonies, higher MIC: 64 µg/ml d) *Aspergillus* sp., higher MIC: 128 µg/mL e) *Aspergillus ficcuum*, ellipse with microcolonies, MIC: 0.016 µg/ml f) *Trichoderma* sp., ellipse with macrocolonies, MIC: 0.50 µg/mL.

Table 2 summarizes the MIC results of Posaconazole antifungal drug for the 50 isolates. >90% of the *Aspergillus* sp., *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus ficuum*, *Penicillium* sp., *Rhizopus Stolonifer* and *Trichoderma* sp. are susceptible to Posaconazole. Antifungal susceptibility data showed only 2 *Curvularia* sp. and 1 *Rhizopus* sp. with very high MIC: >32 µg/mL or no inhibition ellipse was observed to Posaconazole antifungal agent. Conversely, *A. niger* were uniformly susceptible to Posaconazole (MIC_{50} 0.047 µg/mL; MIC_{90} 0.094 µg/mL).

Fluconazole is almost ineffective against most molds (Galuppi *et al.*, 2010). In the current study, high-level resistance (MIC >256 µg/mL) to Fluconazole was observed > 90% of the isolates of fungal species (Table 3). No resistance to Itraconazole was observed for *Aspergillus* sp., *Aspergillus ficuum*, *Penicillium* sp., *Rhizopus* sp. and *Trichoderma* sp. In this study, the susceptibility test result clearly showed that isolates of *Aspergillus niger* and *Aspergillus flavus* were mostly susceptible towards Itraconazole (Table 4).

DISCUSSION

Though some *in vitro* antifungal susceptibility tests are now available (Fernandez-Torres *et al.*, 2001; Karaca *et al.*, 2004; Santos *et al.*, 2001) including CLSI document regarding filamentous fungi (CLSI, 2008, 2010), no simple reference method has been standardised for testing the drug susceptibility of dermatophytes. Etest is a simple, agar-based gradient technique for antifungal susceptibility testing which provides MIC endpoints instead of inhibition zone diameters. We have tried to evaluate the susceptible dose of most common antifungal agent (Posaconazole, Fluconazole and Itraconazole) against different fungi that are dominant in indoor environment.

In this study, one of the dominating indoor fungi *Aspergillus niger* exhibited minute microcolonies and faint hazes after the application of Posaconazole antifungal drug (Fig. 1a). Most *Aspergillus* species

Table 2. Antimicrobial susceptibilities of 50 indoor fungi to Posaconazole antifungal agent

Fungus (no. of isolates tested)	MIC range (µg/mL)	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)
Aspergillus sp. (12)	0.032-0.19	0.094	0.19
Aspergillus niger (15)	<0.002-2	0.047	0.094
Aspergillus flavus (5)	<0.002-4	0.50	1.50
Aspergillus ficuum (3)	<0.002-0.125	0.023	0.094
Curvularia sp. (2)	.002->32	>32	>32
Penicillium sp.(9)	<0.002-0.25	<0.002	0.064
Rhizopus sp. (1)	>32	>32	>32
Rhizopus stolonifer (2)	0.006-0.38	0.006	0.38
Trichoderma sp. (1)	4	4	4

grew well within 24 hours. *Penicillium* sp. showed (Fig. 1b) a macrocolony with clear endpoint of 0.25 μg/mL for Posaconazole antifungal strip. Similarly several micro-colonies were observed (Fig. 1c) within the clear ellipse when the same species of *Penicillium* were tested against Fluconazole strip contradicting the result of *Trichoderma* sp. which was tested with Itraconazole antifungal agent. The growth of particular species were diffused (Fig. 1f) compared to other species.

Table 2, 3 and 4 summarize the *in vitro* susceptibilities of 50 indoor fungi to Posaconazole, Fluconazole and Itraconazole as determined by the Etest methods. The MIC results obtained by Etest method showed that Posaconazole and Itraconazole are very active against most of the species. A study by Pitisuttithum *et al.,* (2005), reported that cerebral aspergillosis achieved a partial clinical response during Posaconazole therapy. Preliminary clinical studies suggest that Posaconazole has a favourable safety and tolerability profile during short- and long-term (6–12 month) treatment (Ullmann *et al.,* 2003; Negroni *et al.,*2003). Torres-Narbona *et al.*, (2007), investigated that the most active drug was Posaconazole, followed by other antifungal drug.

Most fungal strains have high MICs to Fluconazole antifungal drug and there are no intersecting points for susceptibility. Fluconazole is suitable for the treatment of superficial candidiasis, disseminated candidiasis, cryptococcal meningitis, coccidioidomycosis, and cutaneous candidiasis (Vandeputte *et al.,* 2012). Both species (*Aspergillus niger* and *Aspergillus flavus*) exhibited very low level of susceptible to Itraconazole with MIC values 0.25 and 0.004 μg/mL, unlike studies in United Kingdom that found 50%-70% of isolates with high level of azole resistance (MIC >8μg/mL) (Garcia-Effron *et al.,* 2008). Rajendran *et al.,* (2016), reported that *Aspergillus niger* and *Aspergillus fumigatus* were mainly susceptible towards Itraconazole antifungal drug. Also Itraconazole works by slowing the growth of fungi that cause infection. Itraconazole is only indicated for the treatment of onychomycosis, of superficial infections, and in some cases for systemic aspergillosis (Terrell, 1999). The Etest method was used based on the EUCAST method guidelines and according to Denning *et al.,* (1997), Itraconazole antifungal drug can be used as treatment for invasive aspergillosis and gives less gives side effects compared to other antifungal agent. Itraconazole capsules are used to treat fungal infections in the lungs that can spread throughout the body, to treat fungal infections of the toenails, to treat yeast infections of the mouth and throat or of

Table 3. Antimicrobial susceptibilities of 50 indoor fungi to Fluconazole antifungal agent

Fungus (no. of isolates tested)	MIC range (μg/mL)	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)
Aspergillus sp. (12)	128->256	>256	>256
Aspergillus niger (15)	16->256	128	>256
Aspergillus flavus (5)	64->256	>256	>256
Aspergillus ficuum (3)	32->256	>256	>256
Curvularia sp. (2)	0.016->256	>256	>256
Penicillium sp. (9)	16->256	64	>256
Rhizopus sp. (1)	>256	>256	>256
Rhizopus stolonifer (2)	>256	>256	>256
Trichoderma sp. (1)	>256	>256	>256

Table 4. Antimicrobial susceptibilities of 50 indoor fungi to Itraconazole antifungal agent

Fungus (no. of isolates tested)	MIC range (μg/mL)	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)
Aspergillus sp. (12)	0.008-0.50	0.25	0.25
Aspergillus niger (15)	0.016->32	0.25	0.25
Aspergillus flavus (5)	0.004->32	0.004	0.004
Aspergillus ficuum (3)	0.008-0.25	0.008	0.008
Curvularia sp. (2)	0.25->32	>32	>32
Penicillium sp. (9)	0.008-0.25	0.008	0.25
Rhizopus sp. (1)	0.25	0.25	0.25
Rhizopus stolonifer (2)	0.25->32	0.25	0.25
Trichoderma sp. (1)	0.5	0.5	0.5

the esophagus (tube that connects the throat to the stomach).

Ravuconazole is currently under clinical trial stage of antifungal drug development. They possess a wide range of activity, since they are active against *Candida* sp., *Aspergillus* sp., *Fusarium* sp., *Penicillium* sp., *Scedosporium* sp., *Acremonium* sp., and *Trichosporon* sp. and dimorphic fungi, dermatophytes, and *Cryptococcus neoformans* (Sabo *et al.,* 2000*;* Chiou *et al.,* 2000). While new generation triazoles were shown to be more fruitful against *Candida* sp. and *Aspergillus* sp. (Chiou *et al.,* 2000), compared to traditional triazoles their side effects and drug interactions are similar to those noticed with Fluconazole and Itraconazole (Potoski *et al.,* 2002). Such judgement should upgrade the clinician's power to pick the best choice among the available antifungal drugs.

CONCLUSION

The authors concluded that Posaconazole and Itraconazole is the most effective antifungal drug against most of the fungal species compared to Fluconazole because most of the strains showed very high MIC value. This finding helps the visiting physician to guide therapy choice must be the unique decision and responsibility, base judgement on the specific medical history and knowledge of the patient, pharmacokinetics/pharmacodynamics of the antifungal drugs and clinical experience in curing infections caused by the demanding species of fungal pathogen with the antifungal drugs being considered. More research is needed to determine the role of Posaconazole and Itraconazole as a first-line therapy for the treatment of these invasive and deadly fungal diseases.

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