Antifungal Susceptibility Testing of Pathogenic Aeromycoflora Isolated from Kathmandu

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ABSTRACT

Objective: To identify the predominant pathogenic aeromycoflora present in dense areas of Kathmandu, and perform their antifungal susceptibility test.

Methods: Aeromycoflora were isolated by the Gravity Plate method and identified by observing colony morphology and microscopic methods. For pathogenic mycoflora, MIC test was carried out following "Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard. NCCLS document M38-A". The antifungal agents used were Cotrimazole, Fluconazole, Ketoconazole, Itraconazole and Terbinafine available at pharmaceutical companies of Nepal during study period.

Results: *Penicillium spp* (43.8%) was found to be the most predominant aeromycoflora followed by *Cladosporium spp* (35.5%), and pathogenic *Aspergillus species* (21.4%). Among *Aspergillus species*, *Aspergillus niger* (71.7%) was predominant, followed by *A. flavus* (17.1%) and *A. fumigatus* (11.1%). *A. fumigatus* and *A. flavus* were found to be most sensitive towards Itraconazole (MIC range – <0.0625 - 1µg/ml) while *A. niger* to Ketoconazole (MIC- 0.0625 - >32µg/ml).

Conclusion: The finding of this study helped to identify the potent antifungal drugs available against the pathogenic aeromycoflora.

Key words: Aeromycoflora, *Aspergillus* spp, antifungal agents, MIC test, Nepal

INTRODUCTION

The occurrence of airborne mycobiota in outdoor and indoor environments as well as the fluctuations in their numbers and composition are receiving increasing attention within the framework of potential health hazards to both flora and fauna, including humans (Pyrri and Kapsanaki-Gotsi, 2007). Exposure to fungal spores occurs mostly indoor, but outdoor air is an important source of both aeroallergens and pathogens (O'Gorman and Fuller 2008). It is estimated that worldwide deaths attributed to fungal infections (>1 500 000) are as high as those of tuberculosis (1 500 000) (Denning, 2015).

The transmission of fungal spores to the human host is via inhalation. Among the myriad opportunistic fungal pathogens, *Candida albicans* and *Aspergillus fumigatus* cause the most well-known infections. Yet, the growing list of other opportunistic agents is of increasing importance. New and emerging fungal pathogens include opportunistic yeastlike fungi (e.g., *Trichosporon* species), the zygomycetes, hyaline molds (e.g., *Fusarium* and *Scedosporium* species), and a wide variety of dematiaceous fungi (Lass-Flör *et al.*, 2008).

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With the increasing number of immunocompromised individuals, mycoses have also increased in the last two decades, affecting millions of people worldwide. Invasive fungal infections are devastating and the mortality rates with the three most common species of human fungal pathogens are *Candida albicans*, 20%–40% ; *Aspergillus fumigatus*, 50%–90% ; and *Cryptococcus neoformans*, 20%–70% (Butts and Krysan, 2012). Depending on regional distinctions *Aspergillus flavus* and *Aspergillus terreus* are frequently reported as well and there is evidence that these non-fumigatus pathogens are increasingly common etiologic agents (Binder, 2012). In Asia, the Middle East and Africa, *A. flavus* is the cause of a broad spectrum human diseases due to its ability to survive in hot and arid climatic conditions compared to other *Aspergillus* spp. Worldwide, ~10% of cases of bronchopulmonary aspergillosis are caused by *A. flavus* (Rudramurthy et al., 2019).

Shrestha et al. (2010) reported that fungi are most prevalent in Kathmandu, Nepal and broad spectrum antifungal agents are widely used irrespective of antifungal susceptibility testing. With the increasing fungal infections, it has become a prerequisite to perform antifungal susceptibility testing so as to improve the health of patients with fungal infection.

In Nepal, plenty of studies were carried out in pathogenic bacteria with antibiotic susceptibility tests, but only a handful of studies were done in fungi having negligible antifungal susceptibility tests. Hence, the objective of this study is to survey aeromycoflora in a dense area of Kathmandu, identify predominant pathogenic fungi and perform their antifungal susceptibility test.

METHODS

This is a cross sectional study carried out in densely populated and core areas of Kathmandu city within the period of January to June 2012. The processing of the samples was carried out in the Microbiology laboratory of Amrit Science Campus and SANN International College.

Aeromycoflora were collected from four core locations (Ganesh Temple, Bhotahity, Mahaboudha and Bhedasing) of Kathmandu city. From each location, three different sites were selected representing both indoor and outdoor environments. Both indoor and outdoor samples were performed by Gravity Plate method (Colakoglu 1996; Hedayati et al. 2005) in Potato Dextrose Agar (PDA) medium with Chloramphenical. In each spot, two consecutive plates were kept and exposed for 10 minutes in the indoor environment and 5 minutes in the outdoor environment. A control plate (unexposed) was maintained at each site following the same condition as the sample. All the plates were transported to the laboratory as soon as possible and incubated at different temperatures i.e; one plate at 28^0C and another at 37^0C for 4-7 days till the fungal colonies developed. The number of colonies were counted and recorded every day. The isolated colonies were maintained as pure culture by transferring mycelium or spore into PDA medium for the further study. The identification of isolated fungi was done by studying colony morphology and microscopic methods using references [(Subramanian 1971; Barnett and Hunter 1972; Rapper et al. 1973; Barron 1977; Ellis 1985)]. The identified fungi were stored in sterile water at -20°C for further use.

The antifungal susceptibility test was performed for the predominant pathogenic fungi by following Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard. NCCLS document M38-A (NCCLS, 2008). The medium used was RPMI-1640 medium and antifungal agents used were Cotrimazole, Fluconazole, Ketoconazole, Itraconazole and Terbinafine.

RESULTS

The distribution of aeromycoflora in different collection sites showed more than ten different genus of fungi as *Penicillium spp, Cladosporium spp, Aspergillus spp, Alternaria spp, Mucor spp, Rhizopus spp, Trichoderma spp, Helminthosporium spp, Colletotrichum spp, Culvularia spp Fusarium spp* and few unidentified genera. Table (1) represented the predominant non-pathogenic aeromycoflora as *Penicillium* (43.8%) and *Cladosporium* (35.5%) and pathogenic as *Aspergillus* spp (21.4%). Table (1) also showed that indoor environments more polluted by aeromycoflora than outdoor environments.

Table (2) showed that among *Aspergillus* spp, Aspergillus niger (71.1%) was found most prevalent followed by *A. flavus* (17.1%) and *A. fumigatus* (11.1%). In case of resident and non-resident indoor environments, non-residents were more polluted.

Table (3) and (4) showed the MIC value of antifungal agents Ketoconazole and Itraconazole respectively against tested strains of *Aspergillus* spp considering the degree of pathogenicity. For other antibiotics also, MIC was determined following the same procedure.

Table (5) represented the MIC value of all antifungal agents against *Aspergillus* spp. The MIC range differ with *Aspergillus* spp and antifungal agents.

Table 1. Distribution of predominant and pathogenic mycoflora at different site in different environment

Collection site	Environment Type	*Penicillium spp*	*Cladosporium spp*	*Aspergillus niger*	*Aspergillus flavus*	*Aspergillus fumigatus*	*Aspergillus sulphurous*
Ganesh Temple (G)	Outdoor (GO)	20(6%)	9(3%)	8(7%)	3(12%)	3(18%)	1(6%)
	Indoor (GI)	73(21%)	23(8%)	25(23%)	9(35%)	11(65%)	2(13%)
Bhotahity (B)	Outdoor(BO)	6(2%)	4(1%)	2(2%)	0(0%)	0(0%)	0(0%)
	Indoor (BI)	40(12%)	43(15%)	33(30%)	1(4%)	2(12%)	7(44%)
Mahaboudha (M)	Outdoor(MO)	14(4%)	9(3%)	7(6%)	3(12%)	1(6%)	0(0%)
	Indoor (MI)	69(20%)	92(33%)	13(12%)	3(12%)	0(0%)	4(25%)
Bhedasingh (D)	Outdoor(DO)	24(7%)	25(9%)	3(3%)	2(8%)	0(0%)	1(6%)
	Indoor (DI)	94(28%)	74(27%)	18(17%)	5(19%)	0(0%)	1(6%)
Total isolates		340(100%)	279(100%)	109(100%)	26(100 %)	17(100%)	16(100 %)
Grand total	**787**	**43.8%**	**35.5%**	**13.9%**	**3.3%**	**2.2%**	**2.0%**

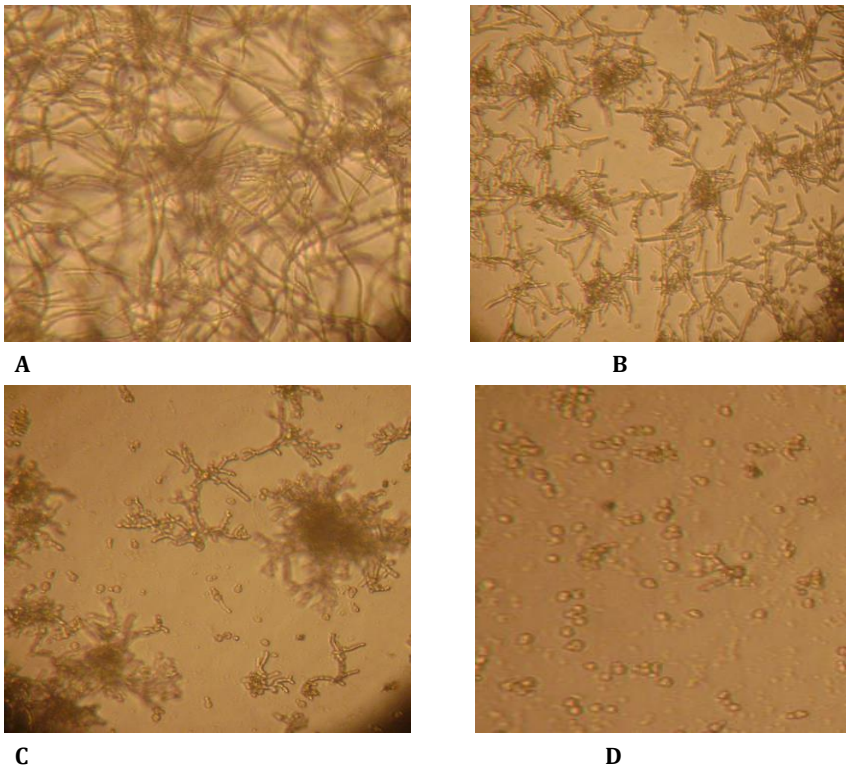
Table2. Distribution of pathogenic fungi at different sites

Collection site	*Aspergillus niger*	*Aspergillus flavus*	*Aspergillus fumigatus*
A. Outdoor			
1. Ganesh Temple	8	3	3
2. Bhotahity	2	0	0
3. Mahaboudha	7	3	1
4. Bhedasingh	3	2	0
Total isolates	20 (18.3%)	8(30.8%)	4(23.5%)
B. Indoor (resident)			
House No. 1	18	1	5
House No 2	4	1	0
House No 3	12	4	0
Total isolates	34(31.1%)	6(23.1%)	5(29.4%)
C. Indoor (non-resident)			
Corporate house 1	1	1	0
Corporate house 2	6	1	0
Nepal Dairy	9	2	0
Subway	32	0	2
Seed shop	7	8	6
Total isolates	55(50.5%)	12(46.2%)	8(47.1%)
Grand Total (152)	**109(71.7%)**	**26(17.1%)**	**17(11.1%)**

:Ptotographs (1-3) of aeromicroflora: 1) Aeromycoflora isolated by gravity plate; 2) *Aspergillus fumigatus* (pure culture); and 3) *Aspergillus fumigatus* (microscopic view)

Table 3. MIC determination of Ketoconazole against *Aspergillus* spp

Ketoconazole concentration (µg/ml)	0 >32	1 32	2 16	3 8	4 4	5 2	6 1	7 0.5	8 0.25	9 0.125	10 0.0625	0 <0.0625	No growth	Total isolates
Aspergillus niger	1	1	2	2	8	2	1	2	2	2	1	0	0	24
Aspergillus flavus	0	1	0	7	6	1	0	0	0	0	0	0	0	15
Aspergillus fumigatus	0	2	8	0	1	1	1	1	0	0	0	0	0	14



A **B**

C **D**

Photograph 4 (A-D): Decrease fungal growth rate in successive higher concentration of antifungal agents in MIC test observed under inverted microscope.

Table 4. MIC determination of Itraconazole against *Aspergillus* spp

Itraconazole concentration (µg/ml)	0 >32	1 32	2 16	3 8	4 4	5 2	6 1	7 0.5	8 0.25	9 0.125	10 0.0625	0 <0.0625	No growth	Total isolate
Aspergillus niger	0	0	0	0	0	0	0	0	0	0	0	0	24	24
Aspergillus flavus	0	0	0	0	0	0	1	2	0	8	0	3	1	15
Aspergillus fumigatus	0	0	0	0	0	0	3	1	0	6	0	3	1	14

Table 5. AST pattern of antifungal agents against *Aspergillus* spp

Antifungal agent and MIC criterion (µg/ml)	*Aspergillus fumigatus*	*Aspergillus flavus*	*Aspergillus niger*
Ketoconazole			
MIC range	0.5 – 32.0	2.0 – 32.0	0.0625 - > 32.0
MIC (max.)	16.0	8.0	4.0
Itraconazole			
MIC range	<0.0625 – 1.0	<0.0625 – 1.0	-
MIC (max.)	0.125	0.125	-
Fluconazole			
MIC range	0.5 - >128	<0.25 - >128	-
MIC (max.)	8	>128	-
Cotrimazole			
MIC range	0.5 – 32.0	2.0 – 32.0	-
MIC (max.)	16.0	8.0	-
Terbinafine			
MIC range	<0.0625 – 0.5	0.125 – 4	-
MIC (max.)	0.25	0.25	-

DISCUSSION

four densely populated sites (Bhedasingh, Bhotahiti, Mahaboudha and Ganesh temple of Ason) were chosen and samples were collected from the outdoor and indoor environments of each site. In the indoor environment, residents and non-residents were considered.

More than ten different types of fungi were isolated in this study. *Penicillium spp* (43.8%) was found to be the most predominant aeromycoflora followed by *Cladosporium* spp (35.5%), and pathogenic *Aspergillus* species (24.1%) as shown by Table 1. Rajbhandari GS (2018) also stated that Aspergilli/ Penicilli group was recorded most predominant with 81.20% and *Cladosporium* as the second highest with its prevalence (8.72%) of the total isolates. The predominance of *Penicillium. Aspergillus* and *Cladosporium* spp were also mentioned by other studies, but differ in most predominant one. Studies by Herrero *et al.* (2006) in Spain and Pyrri and Kapsanaki-Gotsi (2007) in Greece observed *Cladosporium* spp (41% and 37.2% respectively) as most predominant while Shams-Ghahfarokhi *et al.* (2014) in Iran mentioned *Aspergillus* spp (31.3%) as most common one.

Air quality, both indoor and outdoor, is closely linked to a range of diseases, including respiratory, cardiovascular and vascular diseases. Therefore, indoor air pollution becomes

a very important factor affecting the human health (Radwan and Abdel-Aziz, 2019). Table (1) showed that aeromycoflora content in indoor samples are higher than outdoor samples. Similar result was also mentioned in by Radwan and Abdel-Aziz (2019) in study of microbial content of air. According to the pathogenicity, *A. fumigatus* is highly pathogenic followed by *A. flavus* and *A. niger.*

In this study, the distribution of *Aspergillus* spp showed *A. fumigatus* (11.1%) was found in low percent than *A. niger* (71.1%) and *A. flavus* (17.1%) which indicated less harm to human health. Considering the sampling site, highly pathogenic *A. fumigatus* found in higher percent at Ganesh Temple area in both indoor and outdoor environments indicating possible health hazard by fungal diseases residing in those areas than other sites.

Non-residental indoor environments were more polluted due to presence of higher percent of aeromycoflora (Table 2). This implies that working people are more vulnerable to fungal diseases. But this also represented that considering the most pathogenic species *A. fumigatus*, high percent (47.1%) was isolated from non-resident indoor followed by resident (29.4%) and by outdoor (23.5%). This indicates there is chances of increase in fungal diseases if ignored the possible outcomes.

The isolated *Aspergillus* Spp were further processed for antibiotic susceptibility test with the antifungal agents – Cotrimazole, Fluconazole, Ketoconazole, Itraconazole and Terbinafine which were collected from various pharmaceutical companies situated in Kathmandu Valley. Since *A. fumigatus* is the most pathogenic spp, every isolates were proceeded for test while for *A. flavus* and *A. niger*, selected strains representing all collected sites were tested. The MIC was determined as shown in Table 3 to 5. For all antifungal agents, drug concentration range was set as <0.0625 - >32 µg/ml so that it will cover number of concentration tested criteria as mentioned in NCCLS – M38-A guidelines. But in case of fluconazole, concentration upto >128 µg/ml was set as guideline mentioned higher MIC range for fluconazole. Table 3 and 4 showed the MIC value of antifungal agents Ketoconazole and Itraconazole respectively against tested strains of *Aspergillus* spp. For other antibiotics also, MIC was determined following the same procedure. Interpretation of MIC was done following NCCLS-M38-A guidelines. According to guidelines, for Fluconazole, resistant strains shows MIC >64 µg/ml, for Ketoconazole, MIC varies between 0.0313 and 16 µg/ml and for Itraconazole and new Triazoles, MICs >8 µg/ml are associated with clinical resistance to this agent. Espinel-Ingroff *et al.* (2001) mentioned MICs of Itraconazole as >8 µg/ml for resistant and 0.03 to 1 µg/ml for sensitive *Aspergillus* isolates.

Table 5 represented the MIC value of all antifungal agents. In the case of *A. fumigatus*, most sensitive was found to be Itraconazole (MIC – 0.125µg/ml) and least to be Ketoconazole and cotrimazole; in both case MIC - 16µg/ml which lied toward higher range indicating possibility of resistance development. Though Terbinafine also seemed to be sensitive (MIC-0.25 µg/ml), but no growth of isolates in more than 50% of the sample tested was observed.

Denning *et al.* (1997) stated the resistant strain having MIC - >16µg/ml, which is alarming, but in our case no such result appeared. Howard *et al.* (2009) mentioned that the frequency of Itraconazole resistance was 5% in case of clinical isolates, but in this study the result differ as isolates were environmental. In case of *A. flavus,* Itraconazole (MIC – 0.125µg/ml) was found most effective followed by Terbinafin (0.25µg/ml) and resistant to Fluconazole (>128µg/ml). MIC value against *A. niger* was determined for Ketoconazole only (4µg/ml) and no growth was observed against all other antibiotics.

Pfaller *et al.* (2002) mentioned MIC range as 0.25 – 2 µg/ml for *A. fumigatus* and 0.25 – 1 µg/ml for *A. flavus* and Shi *et al.*(2010) determined the MIC as 0.064 - >32 µg/ml for *A. fumigatus* and 0.047 – 4 for *A. flavus* while Mosquera *et al.*(2002) mentioned MIC range for *Aspergillus* spp as 0.125 – 0.5 µg/ml for Itraconazole and 256 -512 µg/ml for Fluconazole. All above mentioned studies were carried with clinical isolates. So, their MIC range lied in higher range than environment isolates of this study.

This study reflected that available antifungal agents were effective for the pathogenic aeromycoflora and proper management of such agents in hospital can decrease resistance development.

CONCLUSION

The predominant mycoflora from different collection sites of Kathmandu city was found to be *Penicillium* spp and potent pathogen, *A. fumigatus*. From the antifungal susceptibility test, Itraconazole was found to be the drug of choice for *A. fumigatus* and *A. flavus* and Ketoconazole for *A. niger*.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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