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In vitro Susceptibility Pattern and Distribution of *Aspergillus* spp. in Hospitalized Patients with Chronic Pulmonary Infection

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Abstract: Aspergillosis is considered as common fungal infection but often seen in immunocompromised patients due to chronic pulmonary infection and other invasive fungal diseases. *A. fumigatus,* the most pathogenic species are responsible for about 90% of all invasive aspergillosis. Though, current treatments are effective but leads to the emergence of resistant strains to commercially available drugs. In the present study sputum samples from different groups of immunocompromised patients was used for the isolation of *Aspergillus* species and screened for multidrug résistance against commercially available drugs such as itraconazole, fluconazole, ketoconazole and amphotericin B. We have isolated 204 *Aspergillus* spp. from different group of immunocompromised patients. *A. fumigatus* was the predominant *Aspergillus* spp (43%) followed by *A. niger* (33%) and others are 24%. Screening of susceptibility pattern of the clinical isolates against antifungal drugs revealed that *A.niger* was resistant to fluconazole (MIC ≥64 mg/L) and itraconazole (MIC ≥32 mg/L) followed by *A. fumigatus* showed resistance to itraconazole (MIC ≥32 mg/L).

Key words: Aspergillosis, immunocompromised patients, multidrug résistance, pulmonary infection

INTRODUCTION

Aspergillus species represents the second most common fungal pathogen in the hospital settings. Aspergillus is a spore-forming fungus that can be found in warm or cold environments, indoors and outdoors. It is thermo tolerant (grows at 15ºC-53ºC) and can thrive in the human respiratory tract. Once inhaled, *Aspergillus* can cause several types of diseases, which depends largely on the underlying immune function of the host. In immunocompetent hosts, *Histoplasma capsulatum*, *Coccidioides immitis*, *Paracoccidioides brasiliensis* and *Blastomyces dermatitidis* are important causative organisms, whereas in immunocompromised patients *Aspergillus*, *Candida*, *Cryptococci pneumocystis jiroveci*, and mucormycosis causing species are important pathogens responsible for causing infections. Immunocompetent individuals rarely develop this type of infection and do so only in the presence of chronic pulmonary and systemic abnormalities such as fibrotic lung disease, (Roselle and Kaufmann, 1978) suppurative infection (Emmons *et al*., 1980) or when they are on corticosteroids (Ng *et al*., 1994).

The immune system normally clears fungal spores, and colonization does not usually cause any significant problems. In patients who have underlying pulmonary disease, especially previous cavity disease as seen in tuberculosis (TB), a fungus ball or aspergilloma may form. Patients who have impaired immunity or chronic lung disease, such as Chronic Obstructive Pulmonary Disease (COPD), can develop semi-invasive or Chronic Pulmonary Aspergillosis (CPA). For those severely immunocompromised, invasive disease can develop and has the potential to disseminate systemically. Other patients can have an exaggerated immune response to *Aspergillus* antigens in the airways and develop Allergic Bronchopulmonary Aspergillosis (ABPA). This is most commonly reported in those who have steroid dependent asthma or Cystic Fibrosis (CF) (Soubani and Chandrasekar, 2002).

The treatment of aspergillosis is difficult and mortality rate remains high despite recent advances in therapy. Three standard antifungal agents are generally employed to treat invasive aspergillosis, including polyenes, nucleoside analogues and azoles. The lipid formulations of amphotericin B appear to be similar in efficacy to conventional amphotericin B in the primary treatment of invasive aspergillosis (Walsh *et al*., 1998; Bowden *et al*., 2002; Walsh *et al*., 2001). The mechanism of action of these drugs leads to development of a variety of adverse events including nephrotoxicity and infusion-related toxicities in a high proportion of patients. Lipid formulations of amphotericin B generally possess a better side-effect profile (Leenders *et al*., 1998; Sundar, 2001) and improved therapeutic index (Maertens *et al*., 2002), but amphotericin B lipid complex and amphotericin B

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colloidal suspension produce a similar incidence of acute infusion-related side effects compared with amphotericin B (Frothingham, 2002; Wingard, 2002). Voriconazole and itraconazole both exhibit non-linear pharmacokinetics, resulting in a greater than proportional increase in exposure with increasing dose (Groll, 2002; Purkins *et al*., 2003) and can give rise to hepatotoxicity necessitating routine liver function testing during therapy. Indeed, it has been estimated that up to 95% of hospitalized patients treated with these agents may receive concomitant agents with the potential of producing a major or moderate pharmacokinetic interaction (Bates *et al*., 2003).

In our present study we have evaluated the pharmacokinetics of four widely used antifungal drugs against the *Aspergillus* strains isolated from patients undergoing treatment for chronic pulmonary infection.

MATERIALS AND METHODS

Study design and sample collection: This was a prospective study on the availability of multidrug resistant *Aspergillus* species among hospitalized patients with chronic pulmonary infections (2007-2009) viz, pulmonary tuberculosis, nasal allergy, chronic pulmonary infection and invasive diseases in the Government Vellore Medical College and Hospital (GVMCH), Vellore, Tamil Nadu, India.

Patients were characterized on the basis of their infection and they were categorized as different group based on underling disease. At least two exporated morning sputum samples on two consecutive days were collected from each patient in a sterile container and were further processed at Biomolecules lab, VIT University, Vellore.

Isolation and characterization: Sputum samples were cultured on Sabouraud's Dextrose agar (Hi Media, India. The positive samples (*Aspergillus*) were separated identified morphologically and at species level on the basis of cultural characteristics. Isolates were stored in glycerol 20% at -70ºC until needed.

Preparation of standard drug stock solution: Different antifungal Amphotericin B (AMB), Itraconazole (ITC), Ketoconazole (KET) and Fluconazole (FLU) were prepared by dissolving the drugs in dimethyl sulfoxide and then diluting further in RPMI-1640 medium. The final concentration range prepared was 0.03-16 µg/ml for AMB, ITC and FLU and 0.15-32 µg/ml for KET (Saurav and Kannabiran, 2010).

***In vitro* antifungal assay:** Antifungal activity with commercially available antifungal drugs viz., Amphotericin B, itraconazole, fluconazole, ketoconazole was determined by using well diffusion, disk diffusion and by the broth two-fold macro dilution standard method CLSI M38-A (formerly NCCLS). The isolates were maintained in 0.2% dextrose medium and the optical density of 0.10 at 530 nm was adjusted using spectrophotometer. Each fungal inoculums were applied on plate and evenly spread on Sabouraud's Dextrose agar (Hi Media, India) using a sterile swab. Well diffusion and disk diffusion assay was followed to evaluate the susceptibility pattern with antifungal drugs. The Petri plates were incubated at 30ºC for 2 days. At the end of the 48 h, inhibition zones formed in the medium were measured in millimeters (mm). All experiments were done in triplicates.

Further the minimum inhibitory concentration of all the isolates were determined by standard protocol. Conidia were cultured on Sabouraud's Dextrose agar (Hi-Media, India) at 30ºC for 2 days. The inoculum was separated on normal saline medium by scrapping the spore by scrapper on the medium, the final inoculums was maintained to $1\text{-}5\times10^4$ colony-forming units/ml. All the drugs were distributed in 96-well, round-bottomed microtitre plates (Nunclon 167008; Nunc, Naperville, IL). The microtitre plates were inoculated with 100 µL of inoculated medium and incubated at 35ºC for 72 h. The MIC was defined as the lowest concentration of drug that produced complete inhibition of fungal growth compared with the growth control. We followed the following breakpoints for all the antifungal, MIC values <2 µg/ml was considered as sensitive, <4 µg/ml as intermediate and >8 µg/ml was resistant and the value >16 µg/ml was considered as MFC value.

RESULTS AND DISCUSSION

Isolation and characterization: Since its first description a little more than half a century ago, Aspergillosis has been reported from all continents and is now seen as an important emerging disease in India (Shah, 1994). The first report from India was in 1971 (Shah, 1971) when a long-term follow-up of three patients was described. Subsequently, several case series were documented (Khan *et al*., 1976; Radha and Vishwanathan, 1978; Chetty *et al*., 1985). Each possible infectious etiology for pulmonary lesions in immunocompromised patients is associated with a significant risk of mortality, as shown in Table 1 *A. fumigatus* was the most common species isolated from adult age group followed by *A. niger* and *A. flavus*. In agreement with the international literature on Indian population survey, we found that *A. fumigatus* was the most common fungal species; accounting for 94.38 and 5.61% of the *Aspergillus* isolates causing infections in adults and children, respectively. *A. niger* was the other species that was isolated from a substantial number of adults (85.29%). The increasing importance of *Aspergillus fumigatus* infection with increasing patient age has been

Table 1: Distribution of *Aspergillus* spp. in samples collected from different group of patients

Disease/Age group	*A. fumigatus*	*A. niger*	*A. flavus*	Others*
Pulmonary TB	48	37	3	5
Lung abscess	8	11	4	0
Lung malignancy	5	4	8	0
Asthma	19	14	9	1
Pleural effusion	3	2	0	9
Bronchopneumonia	6	0	8	0
Pediatric	05	09	03	00
Adult	84	59	29	15
Total	89	68	32	15

*: Others includ pathogenic *Aspergillus* spp. like *A. terrus*, *A. nidulans*

observed in other studies, though not universally documented (Behera *et al*., 1994; Kumar and Gaur, 2000; Chakrabarti *et al*., 2002; Shah *et al*., 2003). Comparison of the prevalence of *Aspergillus* species in sputum shows that it occurs with greater frequency in pulmonary tuberculosis and asthmatic patients than other pulmonary diseases. Occurrence of *Aspergillus* infection in association with chronic lung disease is documented (Edge *et al*., 1971). The isolation of *Aspergillus* from sputum is often considered a contaminant. Whether the isolated agent is a contaminant or indeed responsible for the pathological condition is often questionable?. Routine blood cultures are not much help as they are remarkably insensitive (Kammer and Utz, 1974) and systemic antibody responses in immunocompromised patients are likely to be unreliable indicators of infection (Holmberg *et al*., 1980; Burnie, 1989). However in the present study we have been able to use the routine culture for diagnosis of pulmonary aspergillosis, which resulted in isolation of 204 *Aspergillus* sp.

***In vitro* antifungal assay:** The susceptibility (MIC µg/mL) of 204 *Aspergillus* spp. to amphotericin B, fluconazole, itraconazole and ketoconazole under *in vitro* conditions is given in Table 2. Screening of susceptibility pattern of the clinical isolates against antifungal drugs revealed that *A.niger* was resistant to fluconazole (MIC ≥64 mg/L) and itraconazole (MIC≥32 mg/L) followed by *A. fumigatus* showed resistance to itraconazole (MIC ≥32 mg/L). The percentage of resistance exhibited by the isolates depending on the age of the patient was not statistically significant. Amphotericin B was the most potent antifungal agent among all drugs tested against the Aspergillus isolates. Different prescribing habits to treat children and adults, and horizontal transmission of *A. fumigatus* from patient to patient in the hospital have been considered as contributing factors for the condition observed. The profiles of susceptibility of all species to the drugs tested were generally consistent with those reported previously for *Candida* in both adult and paediatric isolates (Zaoutis *et al*., 2005). No *Aspergillus* spp. may be disregarded with respect to severity of infection. Instead, any *Aspergillus* spp. in air samples from special care areas should raise concern of invasive infection, although certain *Aspergillus* spp. are more commonly involved in nosocomial outbreaks than others. This may, in part, be explained by an increased pathogenicity of this species, but it may also reflect the natural distribution of Aspergillus species in the environment (Shelton *et al*., 2002). To date, the minimal airborne concentration of *Aspergillus* spores necessary to cause infection in patients with significant immunodeficiency remains unknown (Hospenthal *et al*., 1998). Even concentrations of airborne *Aspergillus* spores below 1 colony-forming unit cfu.m^{-3} have been shown to be sufficient to cause outbreaks in immunocompromised patients (Arnow *et al*., 1991). The concordant use of these azole derivative and antifungals among hospitalized patients leads to the development of drug resistant strains more often.

Table 2:Susceptibility of isolated *Aspergillus* spp. against standard antifungal drugs

Species and antifungal agents	No. of isolates	MIC (µg/mL)
A. fumigatus	89	
Amphotericin B		0.125-0.5
Itraconazole		0.25-32
Fluconazole		0.25-4
Ketaconazole		0.25-2
A. niger	68	
Amphotericin B		0.12-0.25
Itraconazole		0.12-32
Fluconazole		0.5- 64
Ketaconazole		0.25-0.5
A. flavus	32	
Amphotericin B		0.5-1
Itraconazole		0.12-0.25
Fluconazole		0.25-0.5
Ketaconazole		<1
***Aspergillus* spp.**	15	
Amphotericin B		0.12-0.5
Itraconazole		0.06-0.25
Fluconazole		0.5-1
Ketaconazole		0.06-0.25

This is our prospective study to find out the multidrug resistant strain among hospitalized Chronic pulmonary infection patients have 11 drug resistant strains among 204 patients studied for a period of a year. The development of drug resistant strain and their handling is a matter of concern. To avoid the outbreak of nosocomial infection with multidrug resistant strain the authors' recommend to follow safety measures for nosocomial infection prevention in accordance with guidelines

published by the Healthcare Infection Control Practices Advisory Committee of the CDC, the Association for Professionals in Infection Control and Epidemiology, the IDSA and the ASBMT (Bartley, 2000).

CONCLUSION

A. fumigatus and *A. niger* are the predominant species in chronic pulmonary infection patients and these strains capable of developing resistance to commonly used antifungal drugs. Hence a suitable measure has to be developed to control the use antifungals and thereby we could bring down the development of multidrug resistance strains in future.

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