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Research Article

SPECIES IDENTIFICATION AND IN VITRO ANTIFUNGAL SUSCEPTIBILITY TESTING OF *ASPERGILLUS* ISOLATED FROM VARIOUS CLINICAL SAMPLES

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ABSTRACT

Objectives: *Aspergillus* is ubiquitous mold species present in environment in the form of spores. Infection due to *Aspergillus* is uncommon in immunocompetent individuals unless they possess any abnormality or have undergone any treatment with corticosteroids. In immunocompromised individuals, infection by this fungus is common among people with prolonged neutropenia, transplants, and human immunodeficiency virus infection. The symptoms are diverse, ranging from allergic reactions to invasive aspergillosis and life-threatening complications. The aim of this study is to isolate and identify *Aspergillus* species obtained from various clinical specimens and perform antifungal susceptibility testing (AFST).

Methods: A total of 200 isolates in which positive direct microscopic findings correlated well with culture growth for *Aspergillus* were included. Antifungal susceptibility was performed by micro broth dilution technique according to clinical laboratory standard institute M38-A2 guidelines.

Results: *Aspergillus flavus* was found to be predominant species followed by *Aspergillus fumigatus*. AFST was performed, where all *Aspergillus* strains exhibited low minimum inhibitory concentration (MIC) and minimum effective concentration (MEC) values for most of antifungal drugs tested except for one strain of *A. flavus*, which exhibited high MIC for voriconazole and high MEC for caspofungin.

Conclusion: Polyenes, azoles, and echinocandin resistance are emerging in many parts of the world. Therefore, continued application of AFST combined with morphological characterization for species identification is critical in detecting the emergence of resistance among various *Aspergillus* species to reduce mortality, morbidity, and overcome treatment failure.

Keywords: *Aspergillus*, Antifungals, Broth microdilution, Lactophenol cotton blue, Slide culture.

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INTRODUCTION

The increased numbers of at-risk patients and enhanced diagnosis have together resulted in an alarming rise in the number of reported fungal infections worldwide [1]. Fungi are a leading cause of morbidity and mortality in cancer, burn, and surgical patients as well as neonatal intensive care unit (ICU) patients [2]. Over the past few decades, filamentous fungi (or molds) have emerged as a major cause of life-threatening infections in immunocompromised patients [1]. Among filamentous fungi, *Aspergillus* is arguably the most important [3].

Aspergilli are saprophytic mold species present ubiquitously in the environment. At present, there are approximately 250 species of this genus, and major species known to cause disease in humans are *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus Niger,* and *Aspergillus terreus* [4].

Aspergillus species can produce infections in both immunocompetent and immunocompromised patients [5]. Individuals with immunodeficiencies due to chemotherapy, organ transplantation, and acquired immunodeficiency syndrome are at higher risk of acquiring infection as compared to immunocompetent individual which show significant defense mechanism against the deadly fungus [6].

Aspergillosis is the name given to all diseases caused by the growth of any member of the genus *Aspergillus* and consists of both invasive and localized infections [7]. *Aspergillus* species primarily cause pulmonary infections with the involvement of other body sites such as paranasal sinuses and cutaneous tissue [6]. *Aspergillus* species cause nervous system aspergillosis among immunocompromised individual whereas paranasal sinus granuloma, keratitis, otomycosis, onychomycoses, cutaneous and wound infections, and osteoarticular infections among immunocompetent individual [1,6,8,9].

Treatment of infections due to these species is really a challenging task as both intrinsic and acquired resistance has been documented. Therefore, emerging antifungal resistance highlights the need for reliable, reproducible, clinically relevant, and sensitive diagnostic tools followed by antifungal susceptibility test to initiate the appropriate antifungal therapy as soon as possible to stem the morbidity and mortality related to aspergillosis [6]. The present study was undertaken to isolate and identify *Aspergillus* species obtained from various clinical specimens and perform antifungal susceptibility testing (AFST).

METHODS

A prospective study was conducted in the department of microbiology in collaboration with chest and TB hospital and other wards of Guru Nanak Dev Hospital, Amritsar, from March 2021 to September 2022. Relevant samples such as ear swabs, nasal discharge, sputum, bronchoalveolar lavage, tracheal aspirate, and corneal scrapping's were processed. Various indoor and outdoor patients clinically suggestive of *Aspergillus* infection were included in the study after approval from the Institutional Ethics Committee.

The samples were processed as per routine mycological procedure. The samples were subjected to direct microscopic examination with potassium hydroxide wet mount and those which showed fungal elements were inoculated on Sabouraud dextrose agar in duplicate. One set of tube was incubated at 37°C and another set at 25°C. Cultures

Table 1: Results of antifungal susceptibility testing among clinical *Aspergillus* isolates

Clinical isolates	Diagnosis		Amphotericin-B (µg/mL)[†]	Itraconazole (µg/mL)	Ketoconazole (µg/mL)	Voriconazole (µg/mL)	Caspofungin (µg/mL) (MEC)[‡]
Aspergillus flavus (n=80)[*]	Otomycosis (3) Fungal sinusitis (28) Ocular aspergillosis (6) Cutaneous aspergillosis (3) Pulmonary aspergillosis (40)	MIC[**] range	0.12–1	0.03–1	0.03–1	0.12–1 ≥2	0.03–0.12 ≥2
Aspergillus flavus		MIC_{50} MIC_{90}	0.25 0.5	0.06 0.5	0.25 0.5	0.25 1	0.06 0.06
Aspergillus fumigatus (n=70)	Otomycosis (1) Fungal sinusitis (2) Ocular aspergillosis (3) Cutaneous aspergillosis (0) Pulmonary aspergillosis (64)	MIC range	0.03–1	0.03–1	0.03–1	0.03–1	0.03–0.12
Aspergillus fumigatus		MIC_{50} MIC_{90}	0.12 0.5	0.06 0.12	0.25 1	0.25 1	0.06 0.06
Aspergillus Niger (n=50)	Otomycosis (45) Fungal sinusitis (1) Ocular aspergillosis (0) Cutaneous aspergillosis (0) Pulmonary aspergillosis (4)	MIC range	0.5–1	0.25–1	0.25–1	0.25–1	0.03-0.12
Aspergillus niger		$MIC^{50[\Pi]}$ $MIC^{90[***]}$	0.5 1	0.25 0.5	0.5 1	0.5 1	0.06 0.06

[†]µg/mL: Microgram/milliliter, [‡]MEC: Minimum effective concentration, *n: number, **MIC: Minimum inhibitory concentration, [Π]MIC_{50}: Antifungal concentration that would inhibit the growth of 50% of tested fungal isolates, ***MIC_{90}: Antifungal concentration that would inhibit the growth of 90% of tested fungal isolates

were examined for growth daily in the 1st week and twice a week for the subsequent period. Macroscopic and microscopic morphology of the obtained growth was studied. The identification of the growth was based on growth rate and colony characteristics such as color, texture, pigment, and submerged hyphae. The speciation of the isolates was done by standard techniques as follows: lactophenol cotton blue tease mount and slide culture technique [6].

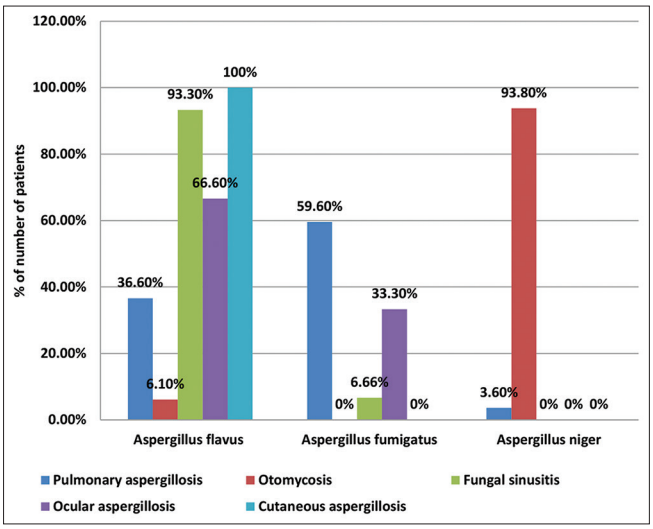
All the isolates obtained were subjected to AFST as per clinical laboratory standard institute M38-A2 guidelines using micro broth dilution techniques for filamentous fungi; approved standard - second edition, intended for testing common filamentous fungi or molds [10]. The five antifungal agents tested were amphotericin B (AMB), itraconazole (ITC), voriconazole (VRC), ketoconazole (KET), and caspofungin (CASP). The concentration ranges tested were 0.03–16 µg/mL (microgram/milliliter) for azoles and AMB and 0.015–8 µg/mL for CASP. American type culture collection strain used was *A. flavus* 204304. The microtiter plate was incubated at 35°C for 24–48 h.

Minimum inhibitory concentration (MIC) was noted for AMB, ITC, VRC, and KET (100% inhibition of growth). Minimum effective concentration (MEC) for CASP was noted, which was the lowest concentration of an antifungal agent that leads to the growth of small, rounded compact hyphal forms as compared to the hyphal growth seen in the growth control well [10].

RESULTS

A total of 200 samples from indoor and outdoor patients were included in the study. The indoor patients constituted 72%, majority of which were cases of pulmonary aspergillosis (24.5%) followed by fungal sinusitis (15.00%), ocular aspergillosis (4.50%), and cutaneous aspergillosis (1.5%) whereas outdoor patients constituted 28%, which included cases of otomycosis (24.5%) and fungal sinusitis (15.00%). Sputum (47%) was the predominant sample followed by ear swab (24.50%), nasal discharge (11.00%), corneal scrapings (8.5%), tracheal aspirate (6.00%), bronchoalveolar lavage (1.00%), pus (1.00%), pleural fluid (0.50%), and gluteal pus (0.50%).

Males (68%) were more commonly affected than females (32%). Total male-to-female ratio was 17:8. The age of the patients ranged



Graph 1: The distribution of *Aspergillus* species among various clinically diagnosed cases

between 11 years and >70 years, with most susceptible age group being 31–40 years (47%), followed by 41–50 years (20%). The mean age±standard deviation of the patients was 39.58±11.17 years. Out of 200 isolates, 79% were immunocompromised with various risk factors such as diabetes mellitus (46.50%), chronic smokers (26.50%), tuberculosis (17%), steroid intake (15.50%), asthma (10%), surgery (9%), ICU stay (9%), hypertension (7.50%), chronic alcoholic (4.50%), chronic obstructive pulmonary disease (3%), and COVID-positive patients constituted 1.5%. Neutropenia (58.50%) was the most common hematological abnormality followed by anemia (38%).

A. flavus was predominant species isolated from 80 (40.00%) out of 200 cases of aspergillosis. *A. fumigatus* was second most common species isolated in 70 (35.00%) cases followed by *A. Niger* in 50 (25.00%) cases. However, *A. fumigatus* was the most common species isolated among cases of pulmonary aspergillosis, *A. flavus* was key species isolated among cases of fungal sinusitis and *A. Niger* was major species isolated from cases of otomycosis as shown in Graph 1.

All *Aspergillus* isolates obtained in this study were subjected to AFST, results of which are depicted in Table 1. All the *Aspergillus* species tested in this study, isolated from suspected cases of aspergillosis exhibited MIC and MEC values in susceptible range except for one species isolated from a case of ocular aspergillosis which was highly resistant to antifungal drugs tested.

DISCUSSION

Within the past few decades, several predisposing factors have increased the incidence of fungal infections, especially with *Aspergillus* species. The various factors include extensive use of antibiotics, warm and humid climate, low socio-economic status with poor hygienic conditions, malnourishment, trauma, surgeries and introduction of new antifungals with variable activity against molds [11].

Aspergillosis infection was predominantly found among males (68%) than females (32%) with male-to-female ratio of 17:8. Most common age group affected was 31–40 years (47%) in all the clinical cases of aspergillosis except for cutaneous aspergillosis in which predominant age group affected was 61–70 years. The similar observations were reported by Kauri *et al.* and Singh *et al.* [12,13]. This may be explained by the fact that males are more involved in outdoor activities and are maximally exposed to fungal spores particularly working with decaying vegetation like moldy hay in agriculture and due to health-seeking behavior among male patients than female patients.

Overall, *A. flavus* (40%) was the predominant species isolated, followed by *A. fumigatus* (35%) and *A. Niger* (25%). The variations in incidence and species distribution could be due to difference in climatic conditions such as temperature and humidity which affect the growth of *Aspergillus* in environment, predisposing conditions present among patients and season of study period.

Most of the *Aspergillus* species were found to exhibit low MIC value in the range of 0.03–1 μg/mL for various antifungal drugs tested: AMB, ITC, VRC and KET, and MEC in the range of 0.03–0.12 μg/mL for CASP, isolated from cases of pulmonary aspergillosis, ocular aspergillosis, otomycosis, and cutaneous aspergillosis except for one strain of *A. flavus*, which exhibited high MIC value of ≥2 μg/mL for VRC and high MEC ≥2 μg/mL for CASP, isolated from a case of ocular aspergillosis, the results of which coincides with the findings of Vishnu *et al.* [7].

CONCLUSION

The incidence of pulmonary and extrapulmonary aspergillosis as well as antifungal drug resistance continues to rise globally among both immunocompromised and immunocompetent individuals. Various infections produced by different *Aspergillus* species are not only difficult to treat but are also difficult to diagnose due to nonspecific signs and symptoms resulting in life-threatening complications. Therefore, precise identification of *Aspergillus* species along with AFST is critical to direct the proper choice of treatment in a timely manner and to abate the emergence of antifungal resistance, and also, to reduce mortality, morbidity, health-care costs in hospitals as well as community.

AUTHOR'S CONTRIBUTION

All the authors contributed equally to concepts, design, data analysis, manuscript preparation, manuscript editing, and review of the article.

CONFLICT OF INTEREST

There are no conflicts of interest.

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