

Clinical profile and azole resistance in clinical and environmental isolates of *Aspergillus flavus* complex from Pakistan

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ABSTRACT

Objective: To determine the azole susceptibility profile of *Aspergillus flavus* (*A. flavus*) complex isolates from clinical and environmental sources in Pakistan, where data on azole resistance are limited.

Materials and Methods: A cross-sectional study was conducted at Aga Khan Hospital, Karachi, from January 2020 to December 2022. A total of 225 *A. flavus* complex isolates (151 clinical and 74 environmental) were included. Minimum inhibitory concentrations (MICs) for voriconazole, itraconazole, and posaconazole were determined using the broth microdilution method in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines. Epidemiological cut-off values (ECVs) were used to identify non-wild-type (NWT) isolates. Clinical data were available for 94 patients.

Results: Invasive aspergillosis was the most common clinical presentation (67/94; 71%), predominantly affecting patients with COVID-19 (35/67). Overall mortality was 32%. One clinical isolate exhibited MICs above the ECV for all three azoles, while two additional clinical isolates showed elevated MICs to posaconazole only. Among environmental isolates, two demonstrated MICs exceeding the ECV for posaconazole. Overall, NWT isolates were identified in 1.98% of clinical and 2.7% of environmental samples.

Conclusion: Azole resistance in *A. flavus* complex remains uncommon in Pakistan; the detection of NWT isolates in both clinical and environmental settings underscores the importance of ongoing surveillance and molecular studies to better characterize resistance mechanisms.

Keywords: Antifungal susceptibility testing, Aspergillosis, *Aspergillus flavus*, Azoles, Drug resistance, Fungal, Epidemiological cut-off values, Pakistan

BACKGROUND

Aspergillus species are opportunistic fungi that cause both allergic and invasive syndromes. Among the wide range of aspergillosis manifestations, invasive aspergillosis (IA) is the most severe one and is linked to high morbidity and mortality rates in immunocompromised patients.¹ The mortality rates are 21% higher in patients having an infection with a voriconazole resistant *Aspergillus* species.² Aspergillosis is most commonly caused by *Aspergillus fumigatus*, followed by *Aspergillus flavus* depending on geographical setting.³ *Aspergillus flavus* complex is more common in Asia, Africa, and the Middle East, and

it is the most common cause of aspergillosis within these regions.⁴ It is reported to be the most common species isolated from clinical specimens in India, a neighboring country to Pakistan.⁵ In Pakistan, *Aspergillus flavus* complex has been reported as the most common agent of aspergillosis from both human and environmental samples.⁶

Broad-spectrum azoles are the first-line antifungal treatment for aspergillosis, while polyenes and echinocandins are sometimes used as alternate or salvage therapy.⁷ Triazole drugs target the 14-sterol demethylase (*cyp51*), an enzyme involved in the production of ergosterol.⁸ However, as resistance to azoles emerges in *Aspergillus* species, treatment of aspergillosis is increasingly becoming difficult. Reported clinical resistance rates differ between countries depending on geographic location and the characteristics of the patient population. A study from Asia found that over 85% of *A. flavus* isolates were resistant to at least one azole.⁹ The acquisition of azole resistance in *Aspergillus* species has been attributed to two different routes: (i) in vivo or in-host acquisition, which can occur after prolonged azole therapy, and (ii)

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acquisition in the environment, which can occur due to the use of demethylation inhibitors (DMIs) fungicides, which have a similar structure to clinical azoles, impose selective pressure on environmental *Aspergillus flavus* strains, driving the emergence of resistant clones.^{10,11}

Although *A. flavus* complex is reported as the most common cause of aspergillosis in Pakistan, data on azole resistance are lacking. Therefore, this study aimed to determine the antifungal susceptibility profile of clinical and environmental *A. flavus* complex isolates in Pakistan.

MATERIAL AND METHODS

It was a cross-sectional study conducted at the Aga Khan University (AKU) Hospital laboratory, Karachi, Pakistan from January 2020 to December 2022.

Sample size estimation for clinical and environmental *A. flavus* complex isolates was performed using the OpenEpi online sample size calculator (Open-Source Epidemiologic Statistics for Public Health).¹² A population size of 500 isolates was assumed. Based on a previous study from Pakistan reporting no azole resistance in clinical *A. flavus* isolates,⁶ an expected azole resistance prevalence of 1% was used for sample size calculation. Using a 95% confidence level, the minimum required sample size was 377 isolates. However, due to isolate availability during the study period, a total of 225 *A. flavus* complex isolates (151 clinical and 74 environmental) were included in the final analysis.

Clinical isolates were collected consecutively from patients presenting to Aga Khan Hospital during the study period, predominantly from those with suspected or confirmed aspergillosis. In addition, a small number of isolates recovered from colonization were included for analysis. Their clinical significance was evaluated using Consensus definitions of the Infectious Diseases Group of the European Organization for Research and Treatment of Cancer and the Mycoses Study Group (EORTC-MSG), Bulpa Criteria, Modified Asp ICU criteria (ASP-ICU), COVID-associated pulmonary aspergillosis (CAPA) criteria, Chronic pulmonary aspergillosis (CPA), Allergic bronchopulmonary aspergillosis (ABPA)-modified ISHAM-ABPA working group criteria, Bent and Kuhn criteria.¹³⁻¹⁹

Environmental strains included in this study were obtained from a previous study conducted by our team.²⁰ Convenience sampling was used for sample collection,

and volunteers were provided with sterile containers used for routine microbiological specimen collection to obtain soil samples from different sites. These volunteers included individuals traveling to different parts of Pakistan. For fungal isolation, soil samples were processed by preparing a suspension in normal saline, a sterile swab was dipped into the suspension, which was then streaked onto Sabouraud Dextrose Agar (SDA) to obtain fungal colonies. *A. flavus* complex isolates were also recovered from air, and hospital environments.

All isolates were saved in glycerol phosphate broth at -80 degree centigrade.

The study included consecutively collected clinical isolates from patients of all age groups diagnosed with clinical aspergillosis, along with a limited number of isolates representing colonization. Environmental isolates were obtained from various sources, including soil from gardens, agricultural fields, and household environments, as well as air and hospital environments. Non-viable, non-culturable, or contaminated isolates, as well as samples falling outside the predefined clinical scope of the study, were excluded.

A 10% potassium hydroxide (KOH) wet mount and Gram stain were prepared directly from the specimen for microscopic examination, and Calcofluor white staining was performed where indicated. Fungal culture was performed using SDA incubated at 25°C and 37°C under aerobic conditions, Sheep Blood Agar (SBA) incubated at 37°C under aerobic conditions, and Mycosel agar and Potato Dextrose Agar (PDA) incubated at 25°C under aerobic conditions. All cultures were incubated for up to 4 weeks. Plates were examined daily during the first week, followed by twice-weekly examination for the subsequent 3–4 weeks. Identification was based on colony and microscopic morphology to the complex level.²¹

Antifungal susceptibility testing was performed using standard broth microdilution techniques as per CLSI M38 guidelines.²² The drug dilution range was 0.03-16 µg/ml for all antifungals which included voriconazole (VOR), itraconazole (ITZ) and posaconazole (POS). Plates were read at 46-50 hours of incubation. The MIC values were interpreted according to established epidemiological cut-off values (ECV): 0.5 µg/ml for POS, 1 µg/ml for ITZ and 2 µg/ml for VOR, given by CLSI M57.²³ The MICs of isolates that exceeded the ECVs were retested for confirmation. ATCC *Aspergillus flavus* complex 204304 was used as a

quality control strain. The MIC ranges ($\mu\text{g/mL}$) for *Aspergillus flavus* ATCC were as: itraconazole (0.25–0.5), posaconazole (0.06–0.5), and voriconazole (0.5–4). The quality control results were within acceptable quality control ranges on all days of testing.

The project was conducted after obtaining exemption from the Ethical Review committee of Aga Khan University, Karachi, Pakistan (ERC# 2019-0438-2659). Clinical information was available for 94 patients. Data were extracted through an online hospital patient care inquiry system, recorded, and entered into Microsoft Excel®. Descriptive statistics, including frequencies and proportions, were computed using Excel. Patient names were not recorded for confidentiality; dual identifiers (specimen number and patient visit ID) were used to avoid inclusion of duplicate isolates.

RESULTS

A total of 225 *Aspergillus flavus* complex isolates were collected, including 151 clinical and 74 environmental. Clinical isolates originated mainly from the respiratory tract. Environmental isolates were predominantly from Sindh ($n = 44$; 59.4%) and Punjab ($n = 26$; 35.1%). Clinical data were available for 94 patients: 12 (13%) had proven IA, and 55 (59%) had probable IA. CAPA

accounted for 37.6% (probable 20.4%, possible 17.2%), ASP-ICU cases 14 (15%; putative 10.7%, probable 4.3%), CPA 2 (2.1%), and ABPA 5 (5.37%); 7.5% were colonizers, Figure-I. Mean age was 51 years, with 57.4% male. Common comorbidities included hypertension (35.4%), diabetes (32.2%), and malignancy (16.1%). Only one patient had prior antifungal use. Mean hospital stay was 10.5 days; ICU admission and intubation occurred in 39 (41.4%) and 37 (39.3%) cases, respectively. Mortality was 32%, Table-I.

Co-isolated organisms with *Aspergillus flavus* complex were assessed. *Pseudomonas aeruginosa* was most common ($n = 16$; 17.2%), followed by *Acinetobacter* species ($n = 13$; 14%). Tuberculosis was associated with 5% of cases. Among molds, *Aspergillus niger* complex was the most frequently co-isolated (Figure-II). MICs of 151 clinical and 74 environmental *Aspergillus flavus* complex isolates were determined against azoles. Most isolates showed low MICs for voriconazole, itraconazole, and posaconazole, with a few non-wild-types (NWT), exceeding ECVs: one clinical isolate for all azoles, two clinical and two environmental isolates for posaconazole only (Image-I, Table-II).

Figure-1: Characteristics of clinical and environmental isolates.

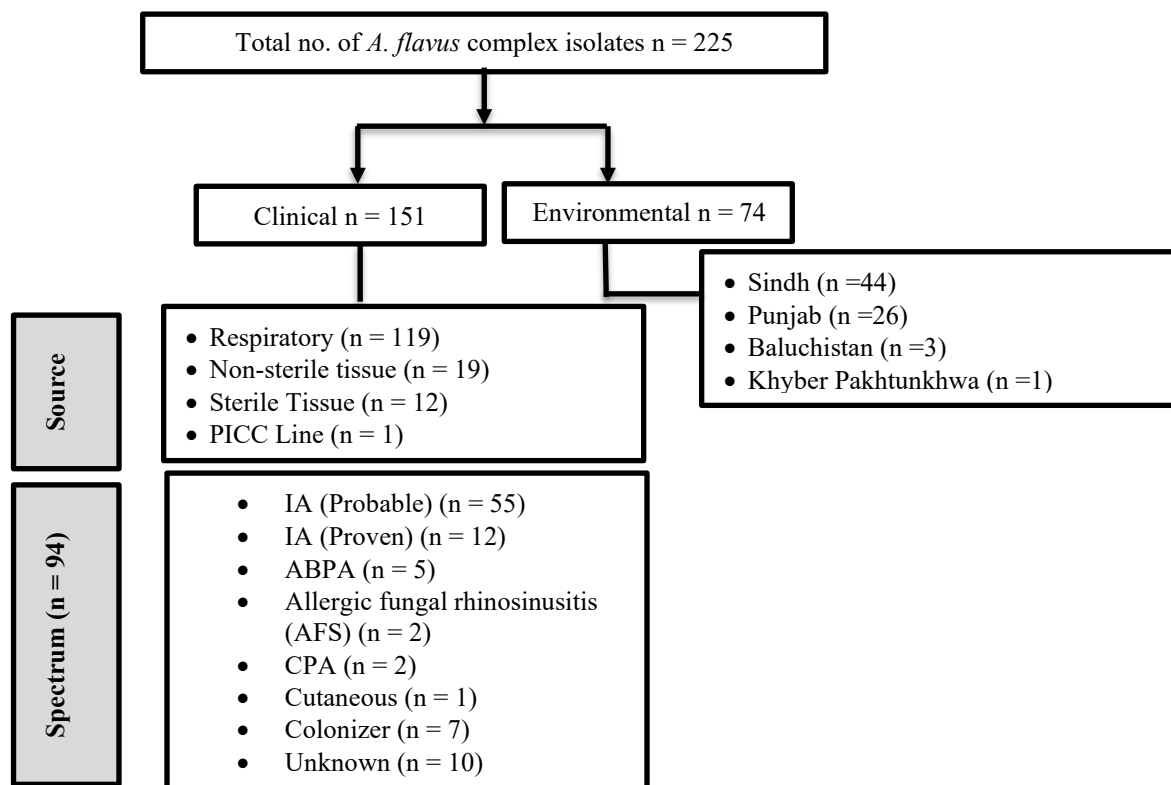


Table-I: Demographics, risk factors, clinical course and outcomes in aspergillosis patients.

DEMOGRAPHICS		Number (%)
Mean Age $\pm$ SD		51.3 $\pm$ 20.8
Male		54 (57.4)
Female		40 (42.5)
COMORBIDITIES AND RISK FACTORS		
Hypertension		33 (35.4)
Diabetes		30 (32.2)
Malignancy		15 (16.1)
Ischemic heart disease		12 (13)
Chronic lung disease		11 (11.7)
Cerebrovascular accident		8 (8.6)
Chronic Kidney disease		6 (6.4)
Autoimmune disorders		6 (6.4)
Chronic liver disease		6 (6.4)
Prior Antifungal Use		1 (1.07)
Others		21 (22.5)
HOSPITAL COURSE		
Average length of stay		10.5 days
ICU Admission		39 (41.4)
Intubation		37 (39.3)
OUTCOME		
Expired		30 (32)
Improved		50 (54.2)
Unknown		13 (13.8)

Table 1 Other comorbidities included central nervous system disorders (6.4%), thyroid disorders (5.4%), benign prostatic hyperplasia (4.3%), dyslipidemia (3.2%), structural heart disease (3.2%). Chronic lung disorders include chronic obstructive pulmonary disease (COPD), asthma, bronchiectasis, and interstitial lung disease.

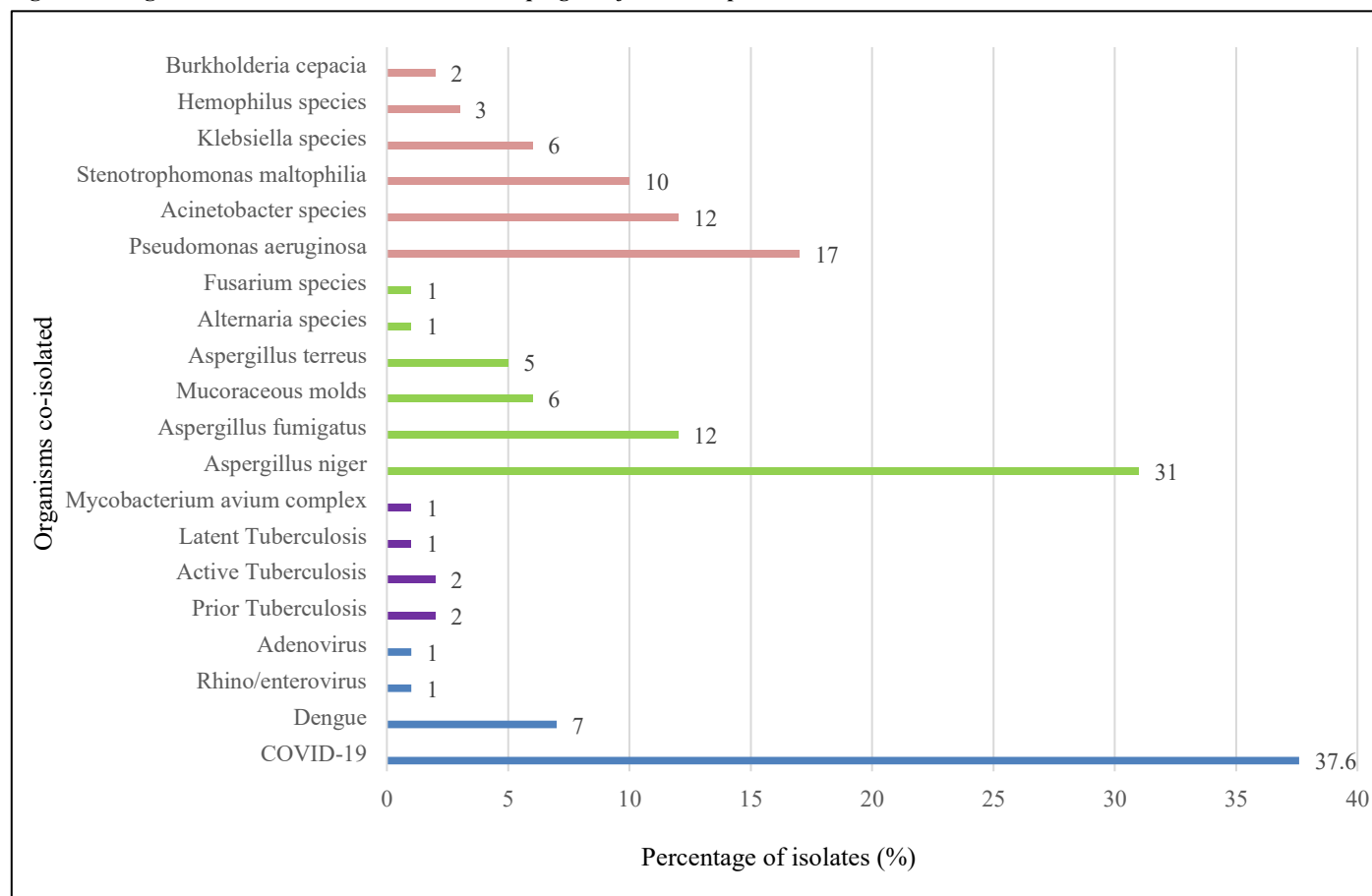
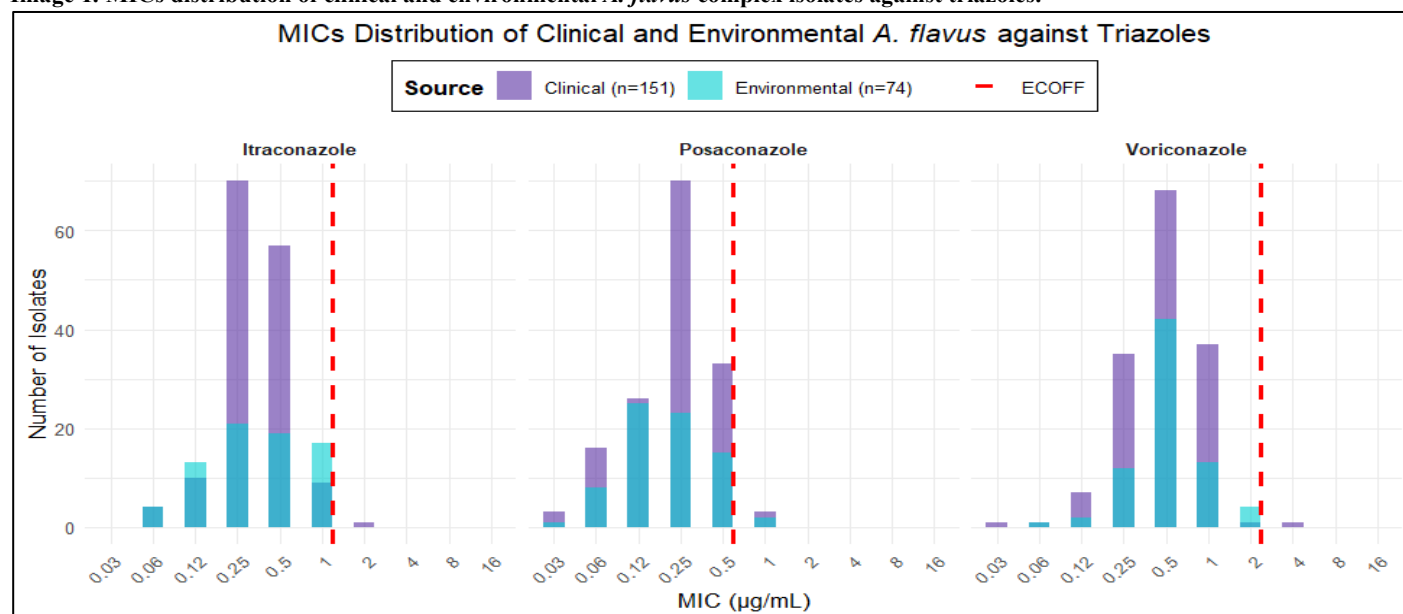
Figure-II: Organisms co-isolated/ identified with *Aspergillus flavus* complex.

Figure 2. Percentage (%) of organisms co-isolated with *Aspergillus flavus* complex from clinical specimens. Co-isolated organisms are grouped as viruses (blue), mycobacteria (purple), fungi other than *A. flavus* (green), and bacteria (pink).

Image-I: MICs distribution of clinical and environmental *A. flavus* complex isolates against triazoles.**Table-II: MIC results for clinical and environmental *Aspergillus flavus* complex isolates.**

CLINICAL n=151					
Antifungal	ECV	MIC range (µg/ml)	MIC ₅₀	MIC ₉₀	No. of isolates above ECV (out of 151)
Voriconazole	2	0.03-4	0.5	1	1
Itraconazole	1	0.06-2	0.25	0.5	1
Posaconazole	0.5	0.03-1	0.25	0.5	3
ENVIRONMENTAL n=74					
Antifungal	ECV		MIC ₅₀	MIC ₉₀	No. of isolates above ECV (out of 74)
Voriconazole	2	0.06-2	0.5	1	0
Itraconazole	1	0.06-1	0.25	1	0
Posaconazole	0.5	0.03-0.5	0.25	0.5	2

DISCUSSION

The susceptibility of a range of clinically significant and environmental *Aspergillus flavus* complex isolates was determined to voriconazole, itraconazole, and posaconazole. Nearly all isolates were found to be susceptible to triazoles, with the exception of one clinical NWT isolate that was non-susceptible to all azoles and three clinical NWT isolates that were non-susceptible to posaconazole. Among the environmental isolates, two strains (2.7%) exhibited MICs above the ECVs. The study finding aligns with a previous study from Pakistan, where triazole resistance was not detected in *Aspergillus* isolates.⁶ A study from Southern Taiwan reported absence of azole-resistant *Aspergillus flavus* complex in two clinical and 16 environmental isolates.²⁴ A study conducted in India revealed resistance in three isolates (2.5%) out of 120 clinical isolates of *Aspergillus flavus* complex.⁵ Similar study from Iran reported *Aspergillus flavus* complex exhibited 1.98% resistance to voriconazole in two out of 101 clinical strains.²⁵ In contrast to findings in this study, a recent study from Tunisia found five (14.7%) and three

(8.8%) of clinical isolates were resistant to itraconazole and posaconazole, respectively, however no isolate was found to be voriconazole resistant.²⁶ Another study from Brazil found azole-resistant *Aspergillus flavus* complex in 7.5% of environmental isolates, which were resistant to itraconazole, but all were susceptible to posaconazole and voriconazole.²⁷ Research from Argentina reported an even higher percentage, with 33.3% of environmental *Aspergillus flavus* complex isolates exhibiting high MIC values for at least one triazole.²⁸

Among 94 patients with clinical data, only one had prior antifungal use. Most *Aspergillus flavus* complex isolates were azole-susceptible. One NWT isolate whose MIC exceeded the ECV for all three azoles, came from a 32-year-old male with a retroperitoneal mass, showing septate hyphae on histopathology; he improved on six-month voriconazole therapy. Two NWT isolates whose MIC exceeded the ECV for posaconazole were recovered from a 31-year-old female with spondylodiscitis (chronic granulomatous inflammation with septate hyphae) and a 36-year-old female with

unknown history. Full clinical details were unavailable for the latter two cases.

This study highlights the spectrum of aspergillosis in clinical settings, with IA being the most common. In Pakistan, IA affects an estimated 3.9% of COPD patients and ~10% of those with acute myeloid leukemia.²⁹ To date, only a few studies have documented the incidence of pulmonary co-infections involving fungi, bacteria and virus.³⁰ Analysis of co-isolated organisms showed *Pseudomonas aeruginosa* as the most frequent bacterium with *Aspergillus flavus* complex. Of these, 31% were associated with COVID-19, highlighting the interplay between viral, bacterial, and fungal infections, consistent with previous studies in critically ill patients.³¹ Excluding COVID-19, 35.2% of aspergillosis cases with *Pseudomonas aeruginosa* occurred in patients with pre-existing pulmonary conditions (ABPA, bronchiectasis, or TB). *Acinetobacter* species (n = 13; 14%) and *Stenotrophomonas maltophilia* (n = 9; 9.7%) were the second and third most common co-isolated bacteria, mostly from hospitalized and intubated patients. These gram-negative bacteria align with previous reports and are commonly associated with hospital-acquired infections.³⁰ In this study, prior and concurrent tuberculosis infections were present in 5% of the aspergillosis patients. Lung damage resulting from TB infection increases the risk of aspergillosis.³² Among the molds co-isolated with *Aspergillus flavus* complex, *A. niger* was the most commonly identified, highlighting the frequent co-existence of polymicrobial fungal infections in clinical settings. Mucoraceous molds were the second most common, with all co-infected patients having diabetes and 100% mortality. Co-infection of aspergillosis and mucormycosis has been reported previously, highlighting its severe clinical impact.³³ Influenza virus detection rates were significantly lower in the pandemic period of COVID-19 compared to the pre-pandemic period as described in a study done at Cameroon.³⁴ Of 94 patients, seven were tested for influenza, all negative. While data on aspergillosis–dengue co-infection are limited, it has been reported in case studies.³⁵ This study included seven patients with dengue fever. Among them, three had concurrent septicemia caused by *Acinetobacter* species, one had *Burkholderia cepacia* complex septicemia. Unfortunately, all these patients succumbed to their illness.

The overall mortality rate in patients was 32%, while the fatality rate among patients with CAPA was 45.7%, which is in line with previously reported high ICU mortality for CAPA, estimated at 54.9%.³⁶ Among 35 CAPA patients on mechanical ventilation, 13 died, with outcomes unknown for three. Severe COVID-19 and ICU admission increase the risk of secondary infections, including aspergillosis.

Evidence on pulmonary fungal co-infections with other pathogens is limited. This study underscores their clinical impact, especially with COVID-19, complicating management and increasing mortality. While we examined co-existing bacteria, fungi, and viruses, the sequence of infection remains unclear.

Study limitations include the small number of strains analyzed and the lack of assessment of resistance mechanisms like CYP51 mutations and efflux pumps due to financial constraints. In addition, molecular speciation of the isolates was not performed.

CONCLUSION

Isolates of *A. flavus* complex with MICs above the ECVs were infrequent among both clinical and environmental isolates; however, the presence of few NWT isolates with elevated MICs highlights the need for ongoing surveillance. Aspergillosis was frequently associated with severe underlying disease and polymicrobial infections, particularly in critically ill and COVID-19 patients, contributing to high mortality. Despite limited isolate numbers and lack of molecular resistance analysis, this study provides important regional data on *A. flavus* complex and emphasizes the importance of continued surveillance.

DISCLOSURES

This article was previously presented as abstract at 11th Congress on Trends in Medical Mycology (TIMM) in October 2023 and ABR2AFR International One Health Stakeholder Meeting on Antifungal Resistance in March 2025

CONFLICT OF INTEREST

None

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AUTHOR CONTRIBUTION

Adan Iftikhar: Substantial contributions to methodology, investigation, validation, analysis, acquisition of data, manuscript writing, critical review, final approval, accountable for all aspects of publication

Tooba Raheem: Data curation, investigation, data analysis, manuscript writing, critical revisions, final approval, accountable for all aspects of publication

Sadaf Zaka: Substantial contributions to methodology, data curation, investigation, data analysis, manuscript writing, critical revisions, final approval, accountable for all aspects of publication

Amna Irfan Ansari: Data curation, investigation, data analysis, manuscript writing, final approval, accountable for all aspects of publication

Joveria Farooqi: Contributed to conceptualization, data analysis, review and editing, final approval, accountable for all aspects of publication

Kausar Jabeen: Contributed to conceptualization, data analysis, supervision, manuscript review, resource provision, final approval, accountable for all aspects of publication

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