



# In Vitro Production of Virulence Factors and Antifungal Susceptibility Pattern of *Aspergillus* Isolates from Clinical Samples in a Tertiary Care Center

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## Abstract

**Objectives** This study was aimed to investigate the association between virulence factors and antifungal susceptibility pattern among *Aspergillus* species.

**Materials and Methods** This study was carried out in the Department of Microbiology, from May 2018 to June 2019. A total of 52 *Aspergillus* isolates obtained from various clinical samples were speciated based on microscopic identification by lacto phenol cotton blue (LPCB) mount and slide culture technique. The production virulence factors such as biofilm, lipase, phospholipase, amylase, and hemolysin were detected using standard phenotypic methods with *Aspergillus niger* ATCC (American Type Culture Collection) 6275 as the control strain. Antifungal susceptibility patterns of all *Aspergillus* isolates to amphotericin B, itraconazole, voriconazole, and posaconazole were evaluated in line with the Clinical Laboratory Standards Institute (CLSI) M38-A2 guidelines.

**Results** The percentage of resistance was the highest in itraconazole (48.08%), followed by amphotericin B (28.85%) and voriconazole (9.62%). All amphotericin B-resistant isolates produced biofilm, itraconazole-resistant isolates exhibited phospholipase activity, and voriconazole-resistant isolates produced biofilm and demonstrated phospholipase and hemolytic activities. Regardless of the virulence factors produced, all isolates were susceptible to posaconazole.

**Conclusion** Understanding the relationship between virulence factors and antifungal resistance aids in the development of new therapeutic approaches involving virulence mechanisms as potential targets for effective antifungal drug development.

## Keywords

- ▶ Antifungal susceptibility
- ▶ *Aspergillus* species
- ▶ biofilm
- ▶ phospholipase
- ▶ virulence factors

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## Introduction

*Aspergillus* species is a filamentous fungus that causes infections in immunocompetent and immunocompromised individuals. Among the recognized species, *Aspergillus fumigatus*, *Aspergillus flavus*, and *Aspergillus niger* are the most encountered etiological agents causing 95% of the disease. The diseases caused by *Aspergillus* in humans are collectively known as aspergillosis. The infections caused by *Aspergillus* species can be localized, allergic, non-invasive, or invasive/disseminated infections. The mortality rate of invasive/disseminated aspergillosis in high-risk groups varies between 40% and 90%, based on factors such as host immune status, site of infection, and appropriate treatment.<sup>1</sup>

Pathogenic *Aspergillus* strains require virulence factors to infect the host. Production of virulence factors by *Aspergillus* species contributes to the establishment of infection, which at a later stage may lead to invasive/disseminated infections.<sup>2</sup> The virulence factors of the pathogen include biofilm production, lipase, phospholipase, amylase, hemolysin, proteinase, esterase, and elastase.<sup>3</sup> Biofilm formation mainly contributes to fungal virulence by promoting the adherence of hyphae to host cells and enhancing resistance to killing by antifungals.<sup>4</sup> A variety of extracellular enzymes produced by *Aspergillus* species break down the complex polysaccharides into simple sugars, which are assimilated and used for growth, reproduction, and the survival of the host organism.<sup>5</sup>

Delay in diagnosis and insufficient information on the virulence of the organism are important contributors for the high mortality in invasive fungal infections.<sup>6</sup> For effective therapy and direct future research, it is also essential to comprehend the association between virulence and antifungal susceptibility profiles of the fungus causing invasive infections. Hence, this study was undertaken to understand the association between production of various virulence factors and the antifungal susceptibility profiles of the *Aspergillus* isolates, which will help to provide an effective treatment.

## Materials and Methods

This hospital-based descriptive study was undertaken in the Department of Microbiology in a tertiary care center after obtaining the institutional ethics clearance. For this study, 52 *Aspergillus* isolates grown from samples of patients collected over 13 months (May 2018 to June 2019) were considered. Repetitive isolates from the same patients were excluded from the study. Basic demographic details were collected from all patients. The strains were maintained in glycerol stock media at 4°C with regular sub-culture for once in 3 months for further testing.<sup>7</sup>

For characterization, the *Aspergillus* isolates were inoculated into Sabouraud dextrose agar (SDA) plates containing gentamycin, and incubated at 25°C for 5 days with regular examination once in 2 days. Once growth appeared in SDA plates, their macroscopic colony morphology was observed. The isolates were systematically identified based on their microscopic morphology on LPCB tease mount and/or slide culture technique.

## Test for Virulence Factors

*Aspergillus niger* ATCC 6275 was the control strain used for testing all virulence factors.

### Biofilm Formation

*Aspergillus* isolates were evaluated for biofilm production by test tube method using 0.1% crystal violet stain.<sup>8</sup> The scoring for tube method was done visually and compared with the results of the control strains. Visible film formation lining the wall and bottom of the tubes was considered to be positive. The appearance of a ring at the liquid interface was not considered as indicative of biofilm formation. The amount of biofilm formed was scored as 0-negative, 1-weak, 2-moderate, and 3-high/strong. The procedure was performed in triplicates.<sup>9</sup>

### Preparation of *Aspergillus* Suspension

Fresh *Aspergillus* isolate (24 to 48 hours) was mixed in sterile saline and the turbidity was adjusted to 0.5 McFarland turbidity standard. Ten microliters of suspension of each isolate was carefully placed on the recommended test medium and incubated at 25°C for 4 to 5 days to evaluate the lipase, phospholipase, amylase, and hemolytic activities. For enzyme production, all strains were tested three times for the enzymatic evaluation and interpreted by two independent observers.

### Lipase Activity

To determine the lipase activity, Tween 80 agar plates with the phenol red indicator medium was used.<sup>10</sup> Lipase activity was considered as positive if there was a change in color from pink to lemon yellow. Transformation of media color from reddish pink to lime yellow was detected due to a change in the pH from basic to acidic (from pH 7.2 to below 6.5) state, caused by the release of the fatty acid due to the degradation of Tween 80.

### Phospholipase Activity

Egg yolk agar plates were used to determine the phospholipase production.<sup>11</sup> The phospholipase activity was marked by the presence of a dense white zone of precipitation around the inoculum. Egg yolk metabolized by phospholipase create precipitation around fungal colonies. The ratio between the colony diameter and the colony diameter plus the halo zone was used to assess the enzymatic activity (Pz) as described by Price et al. The Pz values were classified into four categories based on the scores: elevated Pz values of 1.0 were considered negative, Pz values between 0.999 and 0.700 were considered low enzyme activity (+), Pz values between 0.699 and 0.400 were considered moderate enzyme activity (+ +), and Pz values between 0.399 and 0.100 for strong enzyme activity (+ + +).

### Amylase Activity

The amylolytic activity was determined using the starch agar plate method.<sup>12</sup> Lugol's iodine solution was added to the culture plate after incubation. A clear zone around the

inoculation site was considered as positive for amylolytic activity. Iodine reacts with starch to develop a dark brown color. Hydrolysis of starch will therefore form a clear zone around the inoculum.

### Hemolytic Activity

Hemolytic activity was determined using a 7% sheep blood agar plate.<sup>13</sup> A transparent/semi-transparent zone around the inoculation site was considered as positive for hemolytic activity. By forming pores or holes in red blood cell membranes, hemolysin lyses red blood cells (RBCs), resulting in the release of iron that promotes microbial growth.

### Antifungal Susceptibility Testing

*Paecilomyces variotii* Centraalbureau voor Schimmelcultures (CBS) 132734 was the control strain used for antifungal susceptibility testing.

The antifungal susceptibility testing was done using broth microdilution method, adhering to the CLSI M38-A2 guidelines.<sup>14</sup> The antifungal agents and the range of concentration tested were amphotericin B (A9528-50MG, Sigma-Aldrich, USA): 0.125 to 16 µg/mL.

Itraconazole (16657-100MG, Sigma-Aldrich, USA): 0.125 to 16 µg/mL.

Voriconazole (PZ0005-5MG, Sigma-Aldrich, USA): 0.125 to 16 µg/mL.

Posaconazole (32103-25MG, Sigma-Aldrich, USA): 0.125 to 16 µg/mL.

The results were analyzed and interpreted according to the CLSI guidelines after 48 hours of incubation. As a quality check, the complete absence of turbidity in the media control well was checked each time the test was performed.

### Results

Out of 52 *Aspergillus* isolates obtained from clinical samples, 43 (82.69%) were from ear swab, 4 (7.69%) were from pus, 3 (5.77%) were from tracheal aspirate, and 2 (3.85%) were from bronchial wash.

### Age and Gender Distribution

In this study, percentage of females (69.23%) was more than the males (30.77%), with male to female ratio of 1:2. The age of the patients ranged between 4 and 65 years (mean age: 43 years). Most patients were in the age group 41 to 60 years (42.31%).

### Speciation of *Aspergillus* Isolates

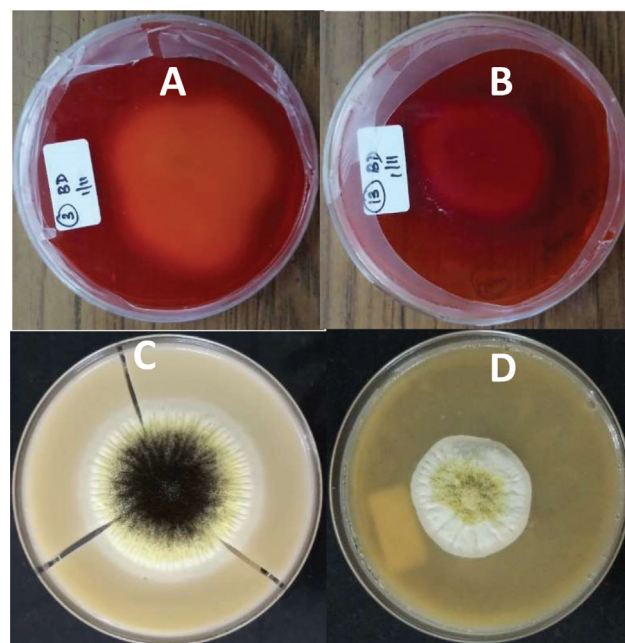
The *Aspergillus* isolates were speciated based on their macroscopic appearance on SDA and microscopic appearance on the LPCB mount.<sup>15</sup> Out of the total 52 *Aspergillus* isolates, 28 i.e., 53.84% isolates were identified as *A. niger*, which is the

most predominant isolate in our study. *A. flavus* was the second-most prominent species isolated in 19/52 isolates, i.e., 36.54%, followed by *A. fumigatus* in 5/52 isolates, i.e., 9.62%.

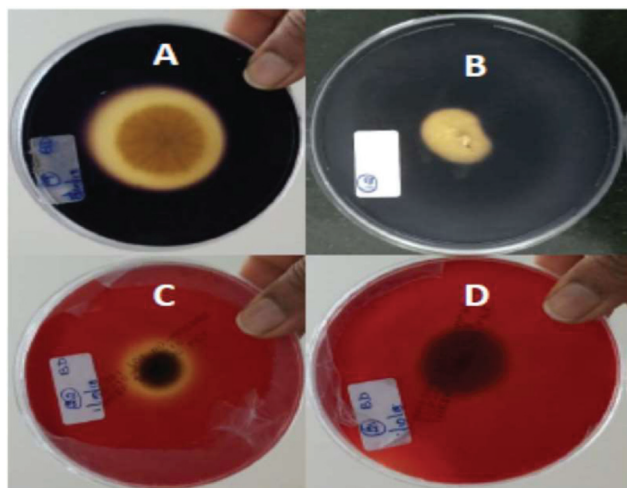
### Production of Virulence Factors

The control strain *Aspergillus niger* ATCC 6275 produced consistent results for all virulence factors tested.

Among the 52 *Aspergillus* isolates tested for biofilm production using tube method, 8 (15.38%) were strongly positive, 12 (23.08%) were weakly positive, and 32 (61.54%) were negative. None of the isolates tested showed a moderate positive result for biofilm production. Out of the 52 *Aspergillus* isolates tested, 25 (48.08%) were lipase positive (►Fig. 1A), and the remaining 27 (51.92%) were negative (►Fig. 1B) for lipase activity. Phospholipase activity was present in 25 (48.08%), out of the 52 isolates tested with a mean Pz = 0.95 (►Fig. 1C). Among these 25 isolates which showed phospholipase activity, 15 (60%) were moderate positive, 10 (40%) were low positive, and 27 (51.92%) were found to be negative for phospholipase activity (►Fig. 1D). None of the isolates tested showed a high production of phospholipase activity. Of the 52 *Aspergillus* isolates, 29 (55.77%) exhibited amylase activity (►Fig. 2A) and 23 (44.23%) were negative for amylase activity (►Fig. 2B). Among the 52 *Aspergillus* isolates, 33 (63.46%) possessed hemolytic activity (►Fig. 2C), and the remaining 19 (36.54%) were found to be negative for hemolytic activity (►Fig. 2D).



**Fig. 1** Lipase activity on Tween 80 agar medium. (A) Lipase positive (arrow showing color change from red to yellow). (B) Lipase negative. Phospholipase activity on egg yolk agar medium. (C) Phospholipase positive (arrow showing a white zone of precipitation). (D) Phospholipase negative.



**Fig. 2** Amylase activity on starch agar medium. (A) Amylase positive (arrow showing a clear halo). (B) Amylase negative. Hemolytic activity on blood agar medium. (C) Hemolysis present. (D) Hemolysis absent.

### Distribution of Virulence Factors among *Aspergillus* species

Among the 52 *Aspergillus* isolates tested for virulence factors, *A. niger* showed the maximum production of biofilm (46.43%), lipase (89.29%), phospholipase (57.14%), and hemolytic (82.14%) activity, whereas amylase activity was observed more in *A. flavus* (89.47%). *A. fumigatus* did not produce any other virulence factor, except amylase activity. The distribution of virulence factors among *Aspergillus* species is shown in (►Table 1).

### Antifungal Susceptibility Testing

For all 52 *Aspergillus* isolates, the mean MIC of itraconazole was found to be higher (6.53 µg/mL) than that of voriconazole (0.93 µg/mL) and posaconazole (0.21 µg/mL) among the triazoles tested in our study. Amphotericin B had the second-highest mean MIC value of 3.77 µg/mL. However, the mean MIC values of *A. fumigatus* was less for amphotericin B (1.15 µg/mL) and itraconazole (0.15 µg/mL) when compared to

other *Aspergillus* species (►Table 2). The resistance percentage was more for itraconazole (48.08%) followed by amphotericin B (28.85%). Posaconazole had 100% sensitivity for all *Aspergillus* isolates. Among the 28 *Aspergillus niger* isolates, 3 (10.71%) isolates were found to be resistant to both itraconazole and voriconazole, and 2 (7.14%) were found to be resistant to amphotericin B and itraconazole. Among 19 *A. flavus* isolates, two (10.53%) were found to be resistant to itraconazole and voriconazole.

### Comparison of Virulence Factors and Resistance Strains of *Aspergillus* Species

All amphotericin B-resistant isolates produced biofilm, itraconazole-resistant isolates exhibited phospholipase activity, while voriconazole-resistant strains produced biofilm and showed phospholipase and hemolytic activities (►Fig. 3). Although *A. fumigatus* is the most common organism that causes invasive aspergillosis, it was found to be less virulent and less resistant. However, *A. niger*, which is the third-most common organism causing invasive aspergillosis, was found to produce most of the virulence factors and was highly resistant to the antifungal agents tested (►Tables 1 and 2). Posaconazole was found to be sensitive regardless of the virulence factors produced by the *Aspergillus* species, hence comparison was not included for the same.

### Discussion

Virulence factors production among *Aspergillus* species could play an important role in the pathogenesis of invasive aspergillosis.<sup>16</sup> Treatment of such invasive infections are difficult if the organism is highly virulent and drug-resistant. In these conditions, comparison of virulence factors and antifungal susceptibility pattern helps the physician to start the patient with a suitable antifungal agent.

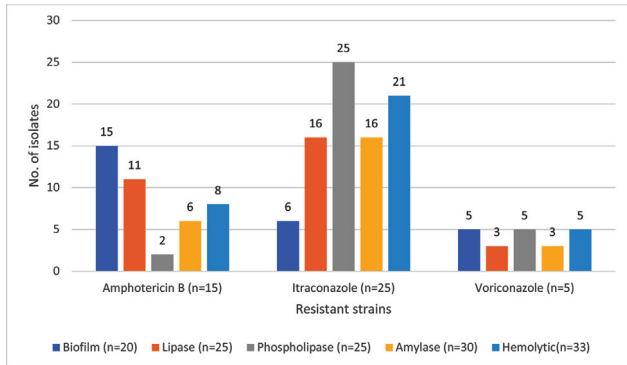
In our study, *A. niger* (54%) was found to be the most predominant isolate, followed by *A. flavus* (37%) and *A. fumigatus* (9%). This finding was similar to a few other studies done by Khaled et al<sup>17</sup> and Satish et al,<sup>18</sup> where *A. niger* was the predominant isolate followed by *A. flavus*. Our finding differed

**Table 1** Distribution of virulence factors among *Aspergillus* species

Virulence factors		<i>A. niger</i> (n = 28)	<i>A. flavus</i> (n = 19)	<i>A. fumigatus</i> (n = 5)
Biofilm	Positive (n = 20)	13 (46.43%)	7 (36.84%)	0
	Negative (n = 32)	15 (53.57%)	12 (63.16%)	5 (100%)
Lipase	Positive (n = 25)	25 (89.29%)	0	0
	Negative (n = 27)	3 (10.71%)	19 (100%)	5 (100%)
Phospholipase	Positive (n = 25)	16 (57.14%)	9 (47.37%)	0
	Negative (n = 27)	12 (42.86%)	10 (52.63%)	5 (100%)
Amylase	Positive (n = 30)	9 (32.14%)	17 (89.47%)	3 (60%)
	Negative (n = 22)	19 (67.86%)	2 (10.53%)	2 (40%)
Hemolytic	Positive (n = 33)	23 (82.14%)	10 (52.63%)	0
	Negative (n = 19)	5 (17.86%)	9 (47.37%)	5 (100%)

**Table 2** Mean MIC values and range of the antifungal agents

<i>Aspergillus</i> species (n = 52)	Mean MIC values/range of the antifungal agents (µg/mL)							
	Amphotericin B		Itraconazole		Voriconazole		Posaconazole	
<i>A. niger</i> (n = 28)	4.69	3.77 (16–0.125)	6.96	6.53 (16–0.125)	0.99	0.93 (8–0.125)	0.26	0.21 (2–0.125)
<i>A. flavus</i> (n = 19)	3.11		7.58		0.97		0.17	
<i>A. fumigatus</i> (n = 5)	1.15		0.15		0.43		0.13	



**Fig. 3** Comparison of virulence factors and resistance strains of *Aspergillus* species.

from another study, where the author found *A. flavus* as the predominant isolate.<sup>19</sup> Hence, it is implied that the distribution of *Aspergillus* species varies by geographical location.

The biofilm production in the present study was seen only among 20/52 (38.46%) isolates. In a previous study by Raksha et al,<sup>20</sup> biofilm production was more, i.e., 32/39 (82%) *Aspergillus* isolates produced biofilm. The test tube method with crystal violet staining could be an acceptable biofilm quantification procedure, but has a high degree of subjective uncertainty, as reading is taken through visual inspection and hence, does not detect moderate to poor biofilm producers accurately.<sup>10</sup> Hence, a better method such as tissue culture plate which is considered to be the gold standard for detection of biofilm production, could be adopted.

In our study, the percentage of production of the enzymes lipase 25/28(89.29%) and phospholipase 16/28(57.14%) was more among *A. niger* compared to *A. flavus* and *A. fumigatus*, which is similar to the findings by Zohri et al,<sup>21</sup> where the maximum number of *A. niger* strains produced the enzymes lipase 27/30 (90%) and phospholipase 30/30 (100%). In our study, among the *Aspergillus* species, *A. niger* had the maximum enzymatic activity which contributes to its role in establishment of infection in susceptible individuals that may later progress to invasive infections in such patients. In the present study, the production of amylase was maximum in *A. flavus* 17 (89.47%), which was in agreement with an earlier study by Fadahunsi et al,<sup>22</sup> whereas this was contrary to the findings of Singh et al,<sup>23</sup> where *A. fumigatus* produced the maximum amylase activity. Diverse sources of carbon and nitrogen can affect the production of amylase.<sup>22</sup> In our study, hemolytic activity was more in *A. niger* (82.14%) followed by *A.*

*flavus* (52.63%), similar to an earlier study by Mezher et al,<sup>13</sup> where *A. niger* produced the maximum hemolytic activity. Hence, in this study, *Aspergillus* isolates were observed to be able to produce an array of extracellular enzymes.

In our study, *Aspergillus* isolates showed high MICs to itraconazole followed by amphotericin B. This was in agreement with a few other studies by Dannaoui et al<sup>24</sup> and Karuthaas et al,<sup>25</sup> where high MICs for itraconazole were observed when compared to amphotericin B. This finding suggest that this drug may not be the right choice to treat invasive aspergillosis. Though voriconazole had a low mean MIC value of 0.93 µg/mL, five (9.62%) of our isolates were resistant to voriconazole. A previous study by Lestrade et al<sup>26</sup> also showed that among 196 patients with invasive aspergillosis 37 (19%) harbored voriconazole-resistant infections. In another study by Sabino et al<sup>27</sup> and Baddley et al<sup>28</sup> high voriconazole mean MIC values were reported for *Aspergillus* isolates. Voriconazole with low mean MIC values to *Aspergillus* isolates can be used to treat patients with aspergillosis. But the problem with this approach is that because there is an emergence of voriconazole resistance being documented globally, in vitro antifungal susceptibility testing is suggested before starting the treatment. Posaconazole with the lowest mean MIC value of 0.21 µg/mL was found to be the most effective antifungal agent when compared to amphotericin B and other azoles. This finding was comparable to a previous report by Sabino et al.<sup>27</sup> Therefore, posaconazole may be considered for the treatment of aspergillosis caused by voriconazole-resistant *Aspergillus* isolates.

A major virulence attribute of *Aspergillus* species, other than antifungal drug resistance, is the production of biofilm that could potentially result in treatment failure and infection recurrence. In our study, all 15 (28.85%) amphotericin B and 5 (9.62%) voriconazole-resistant isolates produced biofilm. These results suggest that higher doses or antifungal combination therapy should be considered for a better penetration of drugs to fungal cells if the isolate is a biofilm producer. Also, in our study, all itraconazole- and voriconazole-resistant isolates exhibited phospholipase activity. In a previous study by Mohammadi et al on *Candida albicans* isolates showed a significant association between high MICs of fluconazole/itraconazole and phospholipase production.<sup>29</sup> Another study by Ghorbel et al had noted a high resistance percentage of amphotericin B among high phospholipase producers of *A. flavus*, whereas our study did not have such correlation between amphotericin resistance and phospholipase

production.<sup>6</sup> The role of virulence in the disease outcome has hardly been considered in aspergillosis, despite the data obtained for other microorganisms.<sup>30</sup>

## Conclusion

Even though virulence factor testing for *Aspergillus* species has been done in many studies, no comparison of virulence factors and antifungal susceptibility testing has been done in India. Though in our study we could establish an association between virulence factor production and antifungal resistance, more research with large sample size may confirm the findings. This warrants their wider surveillance to clearly specify the exact role of virulence factors and drug resistance, especially in patients with invasive aspergillosis. In addition, more genetic and molecular studies may assist in enhanced understanding of the relationship between virulence factors and antifungal resistance, which may help in designing novel therapeutic approaches.

### Note

This study was presented at MICROCON 2018 (01/12/2018).

This study was approved by the institutional ethics committee with ethics clearance number: NI/20/FEB/74/09.

### Funding

None.

### Conflict of Interest

None declared.

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