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Original Article

Microbiology Section

Virulence Factors Detection in *Aspergillus* Isolates from Clinical and Environmental Samples

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ABSTRACT

Introduction: Pathogenesis of aspergillosis is dependent on various factors of the host (immune status) and virulence factors of the pathogen which could play a significant role in the pathogenesis of invasive aspergillosis.

Aim: To study the virulence factors of *Aspergillus* species isolated from patient samples and environmental samples.

Materials and Methods: This prospective and experimental study was carried out at Department of Microbiology, MGM Medical College and Hospital, Mumbai, Maharashtra, India, from July 2014 to June 2015.

For detection of virulence factors of *Aspergillus* species, total 750 samples were included in this study (350 from patients and 400 samples from environment).

Patient samples and hospital environment samples were subjected to standard methods for screening of Biofilm, Lipase, α–amylase, proteinase, haemolysin, phospholipase and pectinase. Statistical analysis was done using Chi-square test and SPSS (Version 17.0).

Results: American Type Culture Collection (ATCC) control of *Aspergillus oryzae, Aspergillus niger* and *Aspergillus brasiliensis* showed production of all virulence factors. In patient samples maximum virulence factor was produced i.e., α-amylase activity (89.74%) followed by proteinase activity (87.17%), biofilm production was (82.05%) haemolysin activity (79.48%), lipase activity (66.66%), pectinase activity and phospholipase activity (61.53%). In environment samples maximum virulence factor was produced i.e., proteinase activity (41.02%) followed by biofilm production was (38.46%), α-amylase activity (35.89%), haemolysin activity (33.33%), lipase activity (28.20%), phospholipase (25.64%) and pectinase activity (23.07%). The differences in patient and environment virulence factors were statistically significant (p-value <0.05).

Conclusion: Overall the presence of virulence factors was found more in *Aspergillus* species isolated from patient samples then environmental samples. This could be due to invasiveness nature of Aspergilli. *Aspergillus niger* was common isolates from both patient and environmental samples. Our study highlights the possible transmission of Aspergilli from environment to patient. Detection of virulence factors of *Aspergillus* species help to differentiate between pathogenic and non-pathogenic Aspergilli. Presence of virulence factors confirmed pathogenicity of the isolates. It also helps the physicians to treat the patient when appropriate treatment is needed.

Keywords: α-amylase, Biofilm, Haemolysin, Lipase, Pectinase, Proteinase, Phospholipase

INTRODUCTION

Aspergillus is a very large genus containing more than 185 species to which human are contantly exposed, only very few of them are responsible for disease. Approximately, 95% of the disease encountered by three species namely *Aspergillus fumigatus, Aspergillus niger* and *Aspergillus flavus.* These are heterotrophic, anamorphic filamentous fungi widely spread in environment; they are common soil inhabitants, also found in large numbers in dust and decomposing organic matter. Under certain conditions, non-pathogenic organisms become pathogenic and are called opportunistic pathogens. Immunocompromised individuals (patients with acute leukemia, transplant recipients, autoimmune diseases, and Acquired Immune Deficient Syndrome (AIDS)) are more prone to this type of infection, *Aspergillus* species are the major cause of high morbidity and mortality which can be present in the human body or acquired by inhalation. *Aspergillus* can grow and reproduce in soil, dust and decomposing organic matter in the presence of nutrients and appropriate temperature, pH and humidity [1,2].

Virulence factors play a major role in the pathogenicity of *Aspergillus* species, host immune status also play a key role for enhancing the production of virulence factors of fungi in host. Other physical factors are also responsible for pathogenicity such as ability of fungus to grow at appropriate temperature, pH of host tissues and small size of conidia [3]. Virulence factors have been identified for different *Aspergillus* species such as adhesins, pigments hydrolytic enzymes such as proteases, phospholipases, ribonucleases, restrictocin; catalases, superoxide-dismutases, mycotoxins and low-molecular-weight non-protein metabolites [4-6]. Mycotoxin mainly gliotoxin is one of the powerful virulence factor, produced by *A. fumigatus* and *A. terreus* isolates in patient samples, however production of aflatoxins are mainly seen in *A. flavus* cases, it is reported that mycotoxins can play a major role to enhance pathogenesis of invasiveness of aspergilli. Also reported that the gliotoxin is produced *in vivo* in tissues of animals infected with *A. fumigatus* [7-10] and it was recently found in serum of patients with invasive aspergillosis [9].

Invasive infections due to filamentous fungi, such as *Aspergillus* species cause significant morbidity and mortality in immuno-compromised patients with haematological malignancies, recipients of haematopoietic stem cell transplants and those with chronic granulomatous disease [11]. Fungi are able to cause diseases and overwhelm the host defense systems because of the presence of several genes and proteins associated with their pathogenicity, called virulence factors. Formation of virulence factors in aspergilli occurrs naturally during their development in human host; these virulence factors protect the aspergilli in unfavourable conditions and enhance the spread of *Aspergillus* infection in tissue [11].

Aspergillosis is associated with wide spectrum of disease which can cause complication during the study of virulence factors such as

some virulence factors associated with invasive aspergillosis which can be irrelevant to other allergic diseases and could be important only in neutropenia or corticosteroid therapy. Aspergillosis infections are usually seen in person with disturbances of the immune system, host factors are as important as fungal virulence factors [12].

The present study was designed to compare the production of virulence factors in *Aspergillus* species isolated from patient and environment.

MATERIALS AND METHODS

This prospective and analytical study was carried out at Microbiology Laboratory, Department of Microbiology, Mahatma Gandhi Mission (MGM) Medical College and Hospital, Navi Mumbai, India, over a period of one year from July 2014 to June 2015. Informed consent was taken from each patient and parent/care taker if age below 18 years before starting the study. Total 350 patients were enrolled for this study out of which males were 232 and females 118 and the age group from 10 years to 70 years with mean age group 40 years, the samples distribution was sputum samples (n=99), nasal and paranasal sinus (n=65), pus samples (n=63), urine (n=51), Bronchoalveolar Lavage (BAL) (n=18), ear (n=29) and eye (n=25). All 350 samples were collected from suspected patients of invasive aspergillosis attending MGM hospital with symptoms of wheezing, shortness of breath, chest pain, cough, headache, weight loss and fever. Patients already on antifungal treatment were excluded from the study. Total 400 environmental samples were taken for the study, the samples were taken from chest TB ward (n=22), medicine ward n=23), ENT ward (n=21), surgery ward (n=13), burns ward (n=17), dermatology ward (n=53), ophthalmology ward (n=34), Medical Intensive Care Unit (MICU) (n=71), Surgical Intensive Care Unit (SICU) (n=78), Intensive Care Unit (ICU) (n=39) and Pediatric Intensive Care Unit (PICU) (n=29). All samples were collected from environment by sterile swabs moisted with sterile peptone water by using template with dimensions 10 × 10 cm [13].

ATCC control strain of *Aspergillus niger* (ATCC® 6275™), *Aspergillus oryzae* (ATCC® 10124™) and *Aspergillus brasiliensis* (ATCC® 16404™) was obtained from Microbiologics Inc, USA.

For detection of virulence factors in *Aspergillus* species in patients samples – 3-5 ml sputum, 10-20 ml urine collected in sterile container, swabs from nasal and paranasal sinuses, pus, ear and eye were collected by sterile swabs and 20-30 ml Bronchoalveolar Lavage (BAL) samples were collected with bronchoscope by trained physicians.

All patient samples (sputum, nasal and paranasal sinuses, pus, ear swab and eye swab and BAL) and swabs from environmental samples (table, light, anaesthesia trolley, floor and wall), were inoculated onto two plates of Sabouraud's Dextrose Agar (SDA) with chloramphenicol; one inoculated plate was incubated at 28°C and the other at 37°C for two to seven days. All isolates were identified by the colony characteristics and morphological appearance. The identification was confirmed by the conidial morphology by preparing slides with one drop of lactophenol cotton blue stain and observed under 40X by light microscope. The identification of *Aspergillus* species was conducted according to Raper KB et al., Ellis D et al., and Afzal H et al., as colonies were detected [14-16]; they were subcultured on Czapeck's Dox Agar (CZA) media for specific species identification according to colony characteristics and microscopical characteristics.

Various virulence factors were studied in *Aspergillus* isolates from patients and environment by standard phenotypic methods such as biofilm formation (by tube method) [17] adherent biofilm layer was scored visually as either negative or weakly (1+), moderately (2+ or 3+), or strongly (4+) positive, lipase production (using T80 agar plates with phenol red) [18], a change in the colour from pink to lemon yellow was observed. α-amylase production (using Starch Agar Plates) [19], observation of hydrolysis, pectinolytic activity (using pectin plates) [20], iodine solution was added to detect the clear zone, proteinolytic activity (using biuret method) [21] recorded the value "x" of unknown from graph corresponding to the optical density reading of the test sample [Table/Fig-1,2], phospholipase activity (using egg yolk agar) [11] the dense white zone of precipitation around the colonies of phospholipase – positive isolates was distinctive and well defined, haemolysis activity (using blood plate assay) was assessed [11]. Similar methods were used for ATCC control of *Aspergillus niger* (ATCC® 6275™), *Aspergillus oryzae* (ATCC® 10124™) and *Aspergillus brasiliensis* (ATCC® 16404™) for detection of different virulence factors [11,17-21].

Ethical Clearance

Ethical clearance was obtained from the Institutional Review Ethics Committee of MGM Institute of Health Sciences, Navi Mumbai, once reviewal of the scientific and ethical consideration of proposal for the clinical study was done. Written informed consent was obtained from each patient separately before starting the project.

STATISTICAL ANALYSIS

Comparison of categorical variables was made by using chi-square test, p-values <0.05 were considered statistically significant. The statistical analyses were performed using SPSS, version 16.0.

RESULTS

Out of 750 samples only in 78 samples showed pure growth of *Aspergillus* species and 672 did not show pure growth, however they showed mixed growth of other fungi and bacteria which was not included in the study. Out of 350 patients samples only 39 *Aspergillus* isolates and from 400 environmental samples only 39 *Aspergillus* isolates were found suitable for the study of virulence factor.

ATCC control of *A. niger*, *A. oryzae* and *A. brasiliensis* showed presence of all virulence factors (biofilm formation, α-amylase production, pectinolytic activity, proteinolytic activity, lipase activity, phospholipase activity and haemolysis activity) which were tested for patients and environmental samples.

Total 78 *Aspergillus* isolates were included for study out of which 39 from patient samples and 39 from environment samples. Maximum number of *Aspergillus niger* (n=38), followed by *Aspergillus fumigatus* (n=18), *Aspergillus flavus* (n=12), *Aspergillus brasiliensis* (n=6) and *Aspergillus terrus* (n=4) were isolated.

In patient samples maximum number of *Aspergillus* species isolated from sputum (n=15) followed by nasal and paranasal sinuses (n=7), pus (n=7), bronchoalveolar lavage (n=3), ear (n=3), eye (n=2), urine (n=2).

In environment samples maximum number of *Aspergillus* species isolated from chest TB ward (n=7), medicine ward (n=7) followed by ENT ward (n=6), surgery ward (n=3), dermatology ward (n=3), opthalmology ward (n=3), MICU (n=3), burns ward (n=2), SICU (n=2), ICU (n=2), PICU (n=1).

Comparison of the production of virulence factors in patient as well as environment samples has been depicted in [Table/Fig-3].

Biofilm production was found in 32 out of 39 patient samples i.e., 82.05% [Table/Fig-4] as compared to environmental samples 15 out of 39 i.e., 38.46%. Lipase activity was seen in 26 out of 39 [Table/Fig-5]. Samples of patients i.e., 66.66% as compared to environmental samples 11 out of 39 i.e., 28.20%. In patients samples α amylase activity was seen in 35 out of 39 [Table/Fig-6] i.e., 89.74% as compared to environmental samples 14 out of 39

Optical Density at 540 nm					
Test Tube No.	*A. niger*	Test Tube No.	*A. fumigatus*	Test Tube No.	*A. brasiliensis*
1.	0.98 (+ve)	19.	0.72 (+ve)	35.	0.88 (+ve)
2.	0.77 (+ve)	20.	0.06 (-ve)	36.	0.78 (+ve)
3.	0.78 (+ve)	21.	0.93 (+ve)	37.	0.99 (+ve)
4.	0.96 (+ve)	22.	0.88 (+ve)	Total Positive	3
5.	0.78 (+ve)	23.	0.95 (+ve)		
6.	0.99 (+ve)	24.	0.81 (+ve)		
7.	1.22 (+ve)	25.	0.83 (+ve)		*A. terrus*
8.	1.02 (+ve)	26.	0.69 (+ve)	38.	0.98 (+ve)
9.	0.03 (-ve)	27.	0.88 (+ve)	39.	0.87 (+ve)
10.	0.79 (+ve)	Total Positive	8	Total Positive	2
11.	0.88 (+ve)				
12.	0.67 (+ve)		*A. flavus*		
13.	0.77 (+ve)	28.	0.87 (+ve)		
14.	0.05 (-ve)	29.	0.66 (+ve)		
15.	0.99 (+ve)	30.	0.05 (-ve)		
16.	0.98 (+ve)	31.	0.75 (+ve)		
17.	0.70 (+ve)	32.	0.64 (+ve)		
18.	0.88 (+ve)	33.	0.88 (+ve)		
Total Positive	16	34.	0.08 (-ve)		
		Total Positive	5		

[Table/Fig-1]: Optical density of proteinase activity of *Aspergillus* isolated from patient samples at 540 nm with spectrophotometry. Cut off value 0.22.

Optical Density at 540 nm					
Test Tube No.	*A. niger*	Test Tube No.	*A. fumigatus*	Test Tube No.	*A. brasiliensis*
1.	0.03 (-ve)	19.	0.54 (+ve)	35.	0.61 (+ve)
2.	0.05 (-ve)	20.	0.03 (-ve)	36.	0.04 (-ve)
3.	0.48 (+ve)	21.	0.62 (+ve)	37.	0.57 (+ve)
4.	0.05 (-ve)	22.	0.05 (-ve)	Total Positive	2
5.	0.52 (+ve)	23.	0.64 (+ve)		
6.	0.48 (+ve)	24.	0.55 (+ve)		
7.	0.04 (-ve)	25.	0.49 (+ve)		*A. terrus*
8.	0.57 (+ve)	26.	0.05 (-ve)	38.	0.59 (+ve)
9.	0.06 (-ve)	27.	0.04 (-ve)	39.	0.03 (-ve)
10.	0.02 (-ve)	Total Positive	5	Total Positive	1
11.	0.03 (-ve)				
12.	0.49 (+ve)				
13.	0.03 (-ve)		*A. flavus*		
14.	0.05 (-ve)	28.	0.56 (+ve)		
15.	0.08 (-ve)	29.	0.05 (-ve)		
16.	0.06 (-ve)	30.	0.02 (-ve)		
17.	0.01 (-ve)	31.	0.49 (+ve)		
18.	0.05 (-ve)	32.	0.43 (+ve)		
19.	0.07 (-ve)	33.	0.01 (-ve)		
20.	0.02 (-ve)	34.	0.02 (-ve)		
Total Positive	5	Total Positive	3		

[Table/Fig-2]: Optical density of proteinase activity of *Aspergillus* isolated from environment samples at 540 nm with spectrophotometry. Cut off value 0.22.

Isolates positive for virulence	Patient's samples (n=39)	Environmental samples (n=39)	p-value significance level <0.05
Biofilm	32 (82.05%)	15 (38.46%)	(0.048) <0.05
Lipase	26 (66.66%)	11 (28.20%)	(0.041) <0.05
α-amylase	35 (89.74%)	14 (35.89%)	(0.017) < 0.05
Pectinolytic	24 (61.53%)	9 (23.07%)	(0.027) <0.05
Proteinolytic	34 (87.17%)	16 (41.02%)	(0.045) < 0.05
Phospholipase	24 (61.53%)	10 (25.64%)	(0.043) < 0.05
Haemolysin	31 (79.48%)	13 (33.33%)	(0.028) < 0.05

[Table/Fig-3]: Comparison of virulence factors in patient and environment samples, p-value calculated by using Chi-square test.

i.e., 35.89%. Pectinolytic activity was seen in 24 out of 39 [Table/Fig-7] patients samples i.e., 61.53% as compared to environmental samples 9 out of 39 i.e., 23.07%. Proteinolytic activity was seen in 34 out of 39 patients [Table/Fig-8] samples i.e., 87.17% as compared to environmental samples 16 out of 39 i.e., 41.02%. Phospholipase activity was seen in 24 out of 39 patients samples [Table/Fig-9] i.e., 61.53% as compared to environmental samples 10 out of 39 i.e., 25.64%. Haemolysin activity was seen in 31 out of 39 patients samples [Table/Fig-10] i.e., 79.48% as compared to environmental samples 13 out of 39 i.e., 33.33%. Comparison of virulence factors in *Aspergillus* species from patent and environmental samples showed, statistically significant (p-value <0.05) [Table/Fig-3].

Biofim production, lipase activity, pectinolytic activity, proteinolytic activity, phospholipase activity and haemolysin activity for all isolates of aspergillus in patient samples and environment samples are shown in [Table/Fig-11,12] respectively.

Virulence factors i.e., biofilm, lipase, α-amylase, pectinase, proteinase, phospholipase and haemolysin in patients and environmental samples showed great variation. In patients samples, the virulence factors showed in maximum number, whereas in environmental samples it presents in less number [Table/Fig-11,12].

DISCUSSION

Various virulence factors – biofilm, proteinase, haemolysin, α amylase, phospholipase, lipase, pectinase were studied. The principle mode of action of these virulence factors is as follows – Various virulence factors cause degradation of tissue carbohydrate (α–amylase), protein (proteinase), phospholipids (phospholipase), pectins (pectinase), and lipids (lipase). Biofilm formation helps prevention of phagocytosis of aspergilli and allows exporential growth [22]. Haemolysin causes lysis of red blood cells. Similarly, cytotoxic and cytolytic lesions are produced by aspergilli.

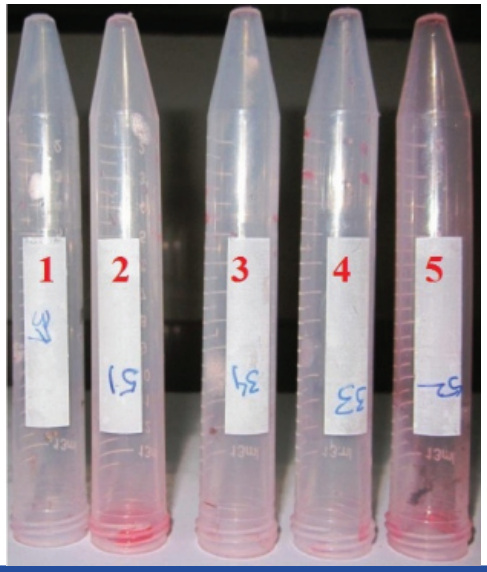
These virulence factors along with toxins initially cause derangement of cellular function, gradually progressing to necrosis/lysis of cells and tissues involving greater portion of organ and finally leading to organ failure.

Study of virulence factors in our work revealed that these virulence factors are present in *Aspergillus* isolates from patients as well as environment.

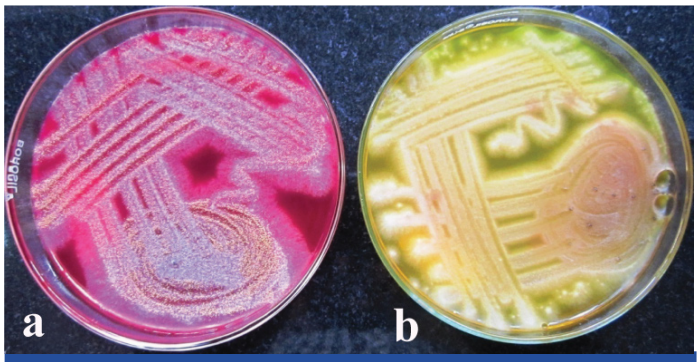
Virulence factors in decreasing order in patient isolates is as follows – α–amylase, proteinase, biofilm, haemolysin, lipase, phospholipase, pectinase.

However, the same decreasing order is not seen in environment samples and number of positivity is also less as compared to patient samples.

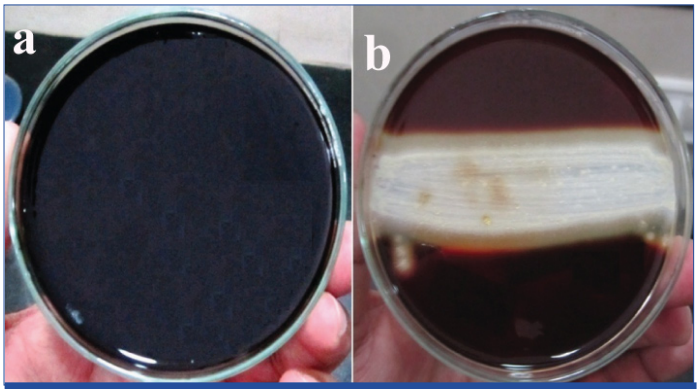
It appears that virulence factors are inherently present in environment samples and active in small percentage for survival in environment. But on entry in human tissues, their activity is enhanced to oppose the adverse condition, protective mechanism of human bodies



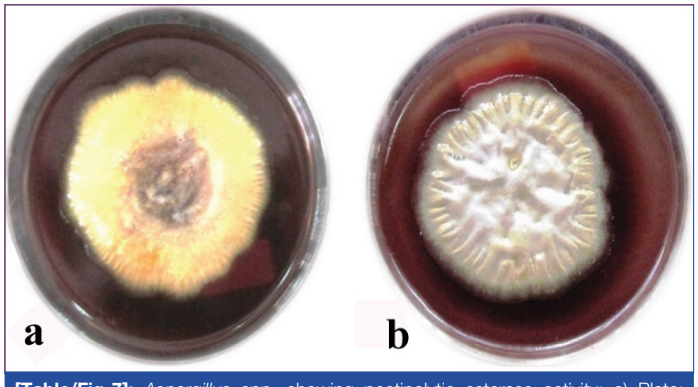
[Table/Fig-4]: Showing biofilm production by *Aspergillus* species, tube 1- negative, Tube 2 to tube 3 weak (+), tube 4 – moderate (2 or 3+) and tube 5 – stong (4+) biofilm production.



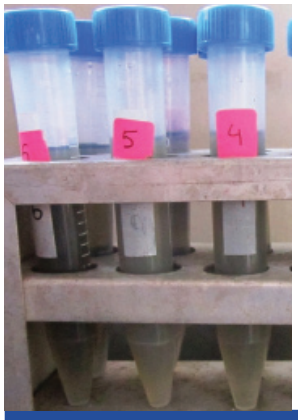
[Table/Fig-5]: Showing lipase activity on Tween 80 agar medium: a) Showed negative for lipase activity; b) Showed positive for lipase activity.

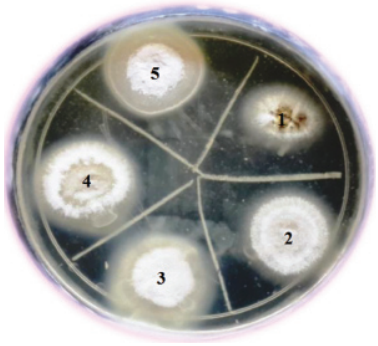


[Table/Fig-6]: *Aspergillus* spp. showing alpha amylase production: a) Plate: control; b) Plate: positive for alpha amylase.

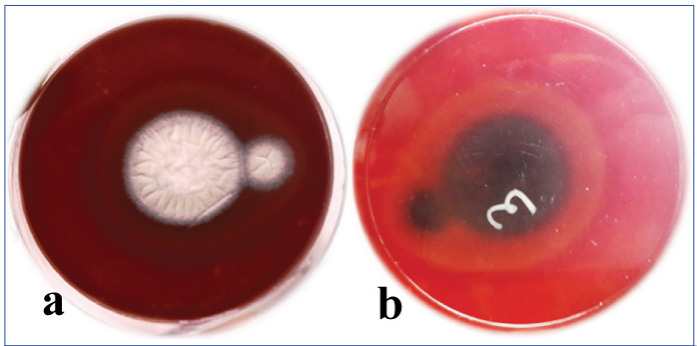


[Table/Fig-7]: *Aspergillus* spp. showing pectinolytic esterase activity: a) Plate: negative for pectinolytic esterase activity; b) Plate: positive for pectinolytic esterase activity.





[Table/Fig-8]: *Aspergillus* spp. showing proteinase activity. Tube 4: control, Tube 5: negative for proteinase activity, Tube 6: positive for proteinase activity; **[Table/Fig-9]:** *Aspergillus niger* showing phospholipase activity. Colony 1: negative control, colony 2: negative for phospholipase activity, colony 3 and 4: positive for phospholipase activity, colony 5: positive control. (Images left to right)



[Table/Fig-10]: *Aspergillus* species showing beta type of haemolytic activity on blood agar. a) Front view of blood agar, b) Back view of blood agar.

which tries to eliminate the microorganisms. Hence, for survival in human tissues, the virulence factors activity is geared up to oppose human protective mechanisms.

Mezher MA et al., studied on 62 patient's sputum samples, out of which 19 (30.6%) were showed growth of *Aspergillus* species [11]. Maximum isolates were *A. fumigatus* (12.9%) followed by *A. terreus* (6.5%), *A. niger* and *A. flavus* (1.6%) each. They reported that *A. fumigatus* showed production of haemolysin and phospholipase 62.5% each and protease production 87.5%, while *A. terreus* showed production of protease and phospholipase 50% each and haemolysin 25%, while *A. flavus*, showed no production of haemolysin, phospholipase protease. All isolates of *A. niger* showed phospolipase production (100%). Gharamah AA et al., sudied on 115 patients samples out of which 25 were *Aspergillus* species isolated, protease activity (80%), lipase (84%) and haemolysis (28%) [23]. Mythili A et al., studied on 108 clinical samples out of which 60 fungal isolates were obtained [24]. Highest lipase activity, protease activity, α-amylase activity and pectinase activity was also prominent for corneal isolate of *Aspergillus* species. Birinci A et al., studied on 30 *A. fumigatus*, nine *A. flavus* and four *A. niger* strains isolated from clinical specimens [25]. *A. fumigatus* showed proteinase 76.7% (23/30) and phospholipase activity 93.3% (28/30). *A. flavus* isolates did not exhibit proteinase or phospholipase activity. *A. niger* showed phospholipase activity (25%) but did not exhibit proteinase activity.

Aspergillus species also play an important role in recyclation of global carbon and nitrogen, and are mainly found in soil or decaying vegetation, the fungi can produce a small, hydrophobic conidia that can be easily circulated and remain present in the air and may survive even an unfavorable environmental condition for a long time, at this stage it can be transferred into human through inhalation. *Aspergillus* contains 200 identified species, out of which 20 or less than that are known to cause human infection [26].

Isolates	No. of isolates	Biofilm Positive	Lipase Positive	α-amylase Positive	Pectinolytic Positive	Proteinolytic Positive	Phospholipase Positive	Haemolysin Positive
A. niger	18	15 (83.33%)	12 (66.66%)	14 (77.77%)	10 (55.55%)	16 (88.88%)	13 (72.22%)	15 (83.33%)
A. fumigatus	9	7 (77.77%)	6 (66.66%)	9 (100%)	5 (55.55%)	8 (88.88%)	5 (55.55%)	7 (77.77%)
A. flavus	7	5 (71.42%)	5 (71.42%)	7 (100%)	4 (57.14%)	5 (71.42%)	3 (42.85%)	4 (57.14%)
A. brasiliensis	3	3 (100%)	2 (66.66%)	3 (100%)	3 (100%)	3 (100%)	2 (66.66%)	3 (100%)
A. terrus	2	2 (100%)	1 (50%)	2 (100%)	2 (100%)	2 (100%)	1 (50%)	2 (100%)

[Table/Fig-11]: Showing species wise production of virulence factors in patient samples.

Isolates	No. of isolates	Biofilm Positive	Lipase Positive	α-amylase Positive	Pectinolytic Positive	Proteinolytic Positive	Phospholipase Positive	Haemolysin Positive
A. niger	20	5 (25%)	4 (20%)	6 (30%)	3 (15%)	5 (25%)	4 (20%)	4 (20%)
A. fumigatus	9	5 (55.56%)	3 (33.33%)	4 (44.44%)	2 (22.22%)	5 (55.55%)	3 (33.33%)	3 (33.33%)
A. flavus	5	2 (40%)	2 (40%)	2 (40%)	2 (40%)	3 (60%)	2 (40%)	3 (60%)
A. brasiliensis	3	2 (66.66%)	1 (33.33%)	1 (33.33%)	1 (33.33%)	2 (66.66%)	1 (33.33%)	2 (66.66%)
A. terrus	2	1 (50%)	1 (50%)	1 (50%)	1 (50%)	1 (50%)	0 (0%)	1 (50%

[Table/Fig-12]: Showing species wise production of virulence factors in environment samples.

Pathogenesis of aspergillosis is dependent on various factors of the host (immune status) and virulence factors of the pathogen. Some putative virulence factors have been identified for different *Aspergillus* species. These include adhesions e.g., biofilm production and haemolysin, pigments hydrolytic enzymes such as proteases, proteinase, lipase, phospholipases, α-amylase, low-molecular-weight, non-protein metabolites [4-6]. These virulence factors could play a significant role in the pathogenesis of invasive aspergillosis [7-10]. Virulence factors of *Aspergillus* species may confirm the pathogenicity and invasiveness nature of fungi. Detection of virulence factor can help to differentiate pathogenic from non-pathogenic aspergilli also help the physican to start antifungal drug whenever needed.

Various immunosuppressive mycotoxins such as gliotoxin are mainly produced by aspergilli. Gliotoxin may produce better under certain culture conditions, such as temperature at 37°C and presence of high oxygen content, which is closely related to the host environment. Gliotoxin is produced in the organs of patients in case of aspergillosis. The present study did not take any account on mycotoxin production. A study reported that the significance of mycotoxins as virulence factors will hopefully be clarified in the near future [27].

LIMITATION

Further studies on large sample size are needed to highlight the roles of various virulence factors to confirm the pathogenecity and invasiveness nature of *Aspergillus* species. Detection of Alpha toxin (aflR gene) in *Aspergillus* isolates by Polymerase Chain Reaction (PCR) may useful for early detection of virulence factors. Studies on gliotoxin are required that may help in the diagnosis of *Aspergillus* associated colonization in context of cystic fibrosis.

CONCLUSION

Virulence factors are very important determinants of any microorganism that help to differentiate pathogenic from non pathogenic species. The presence of virulence factors in aspergilli isolates may give the signal of invasiveness of the *Aspergillus* species. As regards the differences in virulence factors produced by various *Aspergillus* species, it can be seen that protienase, α–amylase, haemolysin, biofilm and phospholipase are most important virulence factors (activity ranges from 42.85% to 100%). These findings are common in *Aspergillus* species isolated from patient and environment samples. This study revealed that aspergilli isolated from environmental samples showed production of virulence factors in less number, but when aspergilli enters into humans, it produces much more virulence factors which can add to the pathogenicity of aspergilli in producing tissue damage.

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