# Prevalence of Aspergillus Infection in Various Clinical Samples with a Special Reference to Black Grain Eumycetoma Caused by *Aspergillus nidulans* in a Tertiary Care Hospital in Eastern India

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**Introduction and study aim:** Mycology has become an extremely challenging study of infections caused by a taxonomically diverse array of opportunistic fungi. Being a widespread soil saprophyte and plant pathogen, Aspergillus species is responsible for superficial and subcutaneous infections, allergic broncho-pulmonary aspergillosis, and another life-threatening invasive aspergillosis. So, proper identification of Aspergillus becomes necessary to ascertain its role in the various infectious etiologies.

**Patients and Methods:** Various clinical samples received in the Mycology lab from November 2022 to May 2023, like nails, urine, respiratory samples, pus, tissue from Functional Endoscopic Sinus Surgery (FESS) and suspected Madura foot were subjected to 10-20% KOH mount followed by fungal culture to confirm the isolate. Slide culture & Banana peel culture were also done as needed. Cultural characteristics & arrangement of phialides & metulae around the vesicle remain the main identifying points to confirm the isolates.

**Results:** Out of 32 (10.7%) Aspergillus sp. from 300 samples, *A. flavus* (28.1%) was the highest followed by *A. fumigatus* & *A. niger* (25.0%); whereas *A. nidulans* & *A. terreus* isolated as lowest (6.2%). Regarding case distribution, the lowest % of cases were found in the 6-12-year age group (3.1%), whereas the highest % of cases were found in the 13–50-year age group (71.9%).

**Conclusion:** Diagnosis of invasive aspergillosis remains difficult clinically due to its wide spectrum of invasion & varied clinical presentation. So, emphasis on fungal culture plays an important role in diagnosing & speciation of Aspergillus infections in various samples.

## INTRODUCTION

Aspergillus species are extraordinary in the context of the diversity of its clinical manifestations. Climatic & geographic conditions may be important determinants of the local prevalence and distribution of Aspergillus sp. The spectrum of Aspergillus infection includes invasive life-threatening infections in immune-compromised hosts, sub-acute or chronic infections in pre-existing pulmonary diseases, allergic manifestations seen in eosinophilic Rhinosinusitis & Allergic Alveolitis, superficial & subcutaneous infections.

This study has been dealt with mainly isolation of Aspergillus sp. from superficial & subcutaneous lesions &systemic samples. Aspergillus sp. has also been shown to be an emerging causative agent of non-dermatophyte (NDM) onychomycosis. Among superficial infections, onychomycosis or nail bed infection is typically caused by dermatophytes (60-70%), whereas NDM contributes to 2-25% cases of Aspergillus accounting for 0.5-3% cases [1]. Common causes & risk factors include hyperhidrosis, local trauma, underlying diabetes, peripheral circulatory diseases, immune suppression & contact with infected household members [2]. Classical signs of onychomycosis commonly include nail discolouration, brittleness, thickening & subungual hyperkeratosis. The main NDM species regarded as causative agents of onychomycosis are; Aspergillus sp., Scopulariopsis sp., Acremonium sp., Fusarium sp., Scytalidium dimidiatum, Onychocola canadensis, Geotrichum candidum etc. [3,4,5].

Among invasive aspergillosis, pulmonary aspergillosis & Rhinosinusitis are most common. Pulmonary aspergillosis mainly affects people with compromised immune status, underlying lung disease or asthma through spore inhalation. Lower WBC count in people having chemotherapy, organ transplant or leukaemia render them more susceptible to invasive Aspergillosis. In this context, BAL fluid is an ideal sample to be processed.

A non-invasive form of paranasal sinus Aspergillosis behaves as chronic rhinosinusitis, manifested as extra-mucosal nasal polypoid mass & are benign in nature. Nasal mass sample with suspected fungal etiology obtained by FESS sent by the ENT department for fungal culture may also give good yield for *Aspergillus* growth. In this study mainly dissected nasal mass has been investigated to conclude mainly non-invasive fungal etiology.

## METHODS

All samples received for Fungal culture in our Mycology laboratory of the Department of Microbiology, NRS Medical College, were studied over a period of six months from November 2022 to May 2023 after abiding by all ethical considerations. After informed consent, taking history and clinical examination of the patient, samples were collected from the lesions as per clinical presentation and order of advice. Samples received were mainly nails, sputum, BAL fluid, pleural fluid, urine, pus, FESS sample, and dissected subcutaneous tissue from the suspected Madura foot.

For nail samples, collection was done after cleaning the infected nail beds with 70% alcohol; nail scrapings & clippings were taken in folded black paper; the nail and FESS tissue samples were subjected to 20% KOH for 10 mins to allow digestion of keratin materials; the other samples like sputum, pus, urine, BAL fluid & pleural fluid were treated with 10% KOH. All KOH wet mounts were then examined under a microscope in 10X followed by 40X for the presence of any fungal elements.

Further, the samples were also inoculated into Sabouraud's Dextrose Agar (SDA) and Sabouraud's Dextrose Chloramphenicol Agar (SDCA) in two sets and incubated at both $25^0$ C & $37^0$ C & examined daily for any growth for 21 days in case of nail and skin, whereas for at least 15 days in case of other samples.

For the urine sample, the centrifuged deposit (1500 rpm for 10 min) was taken into consideration for better yield.

For the FESS sample, a small piece is cut by sterile scalpel blade, placed on a grease-free slide & minced properly. Impression smear of that minced sample is done by rubbing with another slide followed by 20% KOH wet mount.

**Processing of Black Grains in suspected Madura foot** : Dissected tissue section ( left leg) of a 53 years old suspected Madura foot patient were sent to our laboratory from Plastic surgery Dept. Macroscopically the tissue sample was embedded with multiple black grains.

Black grains were isolated from the dissected tissue mass by the tip of the scalpel blade, saline washed several times. A few grains were placed on a grease-free glass slide, covered with a sterile coverslip & crushed by a gentle tap with the help of the back of the inoculation loop handle. Then two drops of 20% KOH were poured by the side of the coverslip & allowed to percolate by capillary action and examined under 10X & 40X. Saline-washed black grains were inoculated in SDA, and SDCA media, incubated at both $25^0$ C & $37^0$ C. Black-colored concentric rough growth was evident after 10-15 days.

**Banana Peel Culture from Growth of Madura foot sample**: In the Banana peel culture method, ripe banana peels were autoclaved at $121^0$ C, 15 lb pressure for 15 mins. After cooling, the peels were placed on sterile Petri plates with the inner side peels outward facing. Colony from SDCA inoculated on the peel at several places & covered with sterile coverslips. Some sterile water was sprinkled on the peels & kept at room temperature. After 15 days coverslip is carefully placed on LPCB on a grease-free slide by forceps, avoiding bubbles & viewed under 40X[6].

Any growth in SDA and SDCA media were studied by their colony morphology, presentation, pigmentation of reverse & obverse surfaces followed by LPCB teased mount and Slide culture wherever necessary.

A. flavus was identified by Sterigmata covering $3/4^{th}$ of Globose or subglobose vesicle, along with phialides & setulae as demonstrated by

LPCB wet mount; *A. fumigatus* showed Globose to sub-globose vesicle having uniseriate phialides; arranged parallel to conidiophore; *A. niger* was diagnosed by Globose vesicle producing brownish-black sterigmata with conidia on its entire surface; *A. Amsterdam* was diagnosed by Flask-shaped vesicle having primary series of sterigmata; V-shaped furrow at one end of cleistothecium (ascosporic state); *A. nidulans* was confirmed by Septate hyaline hyphae, bi-seriate sterigmata over spherical vesicle & characteristic Hulle cells whereas *A. terreus* was identified by bi-seriate sterigmata, alleuro conidia directly arising from conidiophore.

The data obtained were summarized by counts & percentages using Microsoft Excel and other statistical parameters as appropriate.

## RESULTS

In this study among 300 samples tested, 32 isolates have been confirmed as *Aspergillus sp*. Nail & skin scraping accounted for most of the isolates (28.1%) with hand nails having more incidence than toenails revealing mostly Total Dystrophic Onychomycosis (TDO), Proximal Subungual Onychomycosis (PSO) & Distal Lateral Subungual Onychomycosis (DLSO); whereas significant numbers of isolates are diagnosed in BAL fluid & Urine (15.6 % each) followed by pleural fluid (6.2%), sputum (6.2%) & FESS samples (12.5%) and one dissected tissue from Madura foot (3.1% ) (Table I).

The Age-wise distribution showed lowest percentage (3.1%) of cases among 6-12 yr age group whereas highest percentage (71.87%) of cases seen in 13-50 year age group (Table-II).

Gender-wise Male female distribution was equal with 16 cases (50%) each among which 13 (40.6%) cases were adult males & 3 cases (9.37%) were pediatric males whereas 14 cases (43.76%) were adult females & 2 cases (6.25%) were pediatric female.

The details of the prevalence of isolated Aspergillus species revealed *A. flavus* (Fig. I) isolated as highest number 9 (28.1%) followed by *A. fumigatus* 8 (25.0 %) (Fig. II) & *A. niger* 8 (25.0 %) (Fig III); whereas *A. nidulans* 2 (6.2 %) (Fig. IV), *A. Amsterdam* 3 (9.3%) (Fig. V) & *A. terreus 2 (6.2%)* (Fig VI) among total 32 isolates (Table-III).

The suspected Madura foot sample containing black grains was crushed and a 20% KOH mount was prepared which revealed a hyphal structure projecting from the periphery of the crushed grain; the LPCB mount from the culture in SDCA media revealed septate hyaline hyphae, long conidiophores with terminal hemispherical vesicles having double series of sterigmata characteristic Hulle cells diagnostic of *A. nidulans*. LPCB of the coverslip from banana peel culture revealed the same morphology of *A. nidulans* (Table-IV, Fig. VII).

In this study prevalence of Aspergillosis is being 10.7% among all 300 samples processed. No *Aspergillus* sp. was isolated from eye samples in this period of study.

**Table (1).** Samples-wise Clinical Presentation & Prevalence of Aspergillus isolates (n=32)

| Type of sample | Clinical presentation | Fungal isolates | No. of Cases (%) |
|---|---|---|---|
| 1a. Skin Scrapings<br><br>1b. Toenails & Hand nails | Rough skin, often greyish-black, with scaly lesions<br>*TDO, PSO & DLSO types identified | A. niger, A. flavus<br>A. niger,<br>A. fumigatus,<br>A. flavus | 9 (28.1%) |
| 2. FESS sample | Nasal polypoid mass | A. flavus,<br>A. fumigatus,<br>A. niger | 4 (12.5%) |
| 3. Urine | Turbid | A. amstelodami, A. niger,<br>A. fumigatus | 5 (15.6%) |
| 4.Pleural fluid | Little turbidity | A. flavus,<br>A fumigates,<br>A. terras | 2 (6.2%) |
| 5. BAL fluid | Little turbidity | A.flavus,<br>A fumigates,<br>A. terras | 5 (15.6%) |


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| | | | |
|---|---|---|---|
| 5. Pus | Mainly from wound (in 1 case pus collected from non-healing discharging sinus from keloid on anterior chest wall | A. nidulans | 3 (9.3%) |
| 6. FNAC lung lower lobe | Tissue sample | A. fumigatus | 1(3.1%) |
| 7. Fungal ball & Sputum | Dark brown hard fungal mass | A. niger | 2 (6.2%) |
| 8. Dissected tissue of Madura's foot from plastic surgery (53 yr Male) | black grains embedded in a dissected tissue sample | A. nidulans | 1 (3.1%) |
| 9. Ocular Samples | | No Aspergillus | Nil |

*TDO - Total Dystrophic Onychomycosis, PSO - Proximal Subungual Onychomycosis , DLSO - Distal Lateral Subungual Onychomycosis

**Table (2).** Age-wise distribution of Aspergillosis (Total 32 isolates among 300 samples) (n=32)

| **Age group** | **Number of Aspergillus isolates** | **% of distribution** |
|---|---|---|
| **0 -5 yrs** | **2** | **6.2 %** |
| **6 – 12 yrs** | **1** | **3.1 %** |
| **13 – 50 yrs** | **23** | **71.9 %** |
| **>50 yrs** | **6** | **18.8 %** |

**Table (3).** Prevalence of various Aspergillus isolates (N=32)

| **Isolate** | **Number of isolates** | **%** |
|---|---|---|
| **A. flavus** | 9 | 28.12% |
| **A. Fumigatus** | 8 | 25% |
| **A. niger** | 8 | 25% |
| **A. nidulans** | 2 | 6.25% |
| **A. Amsterdam** | 3 | 9.38% |
| **A. terreus** | 2 | 6.25% |

**Table (4).** Findings of Madura foot isolate (Aspergillus nidulans)

| **Procedure** | **Findings** |
|---|---|
| 20% KOH mount in 40X | crushed grains revealed a hyphal structure projecting from the periphery of the crushed grain |
| LPCB from SDCA growth | The features include; septate hyaline hyphae, long conidiophores with terminal hemispherical vesicles having double series of sterigmata , characteristic Hülle cells. Some conidiophores showed sinuous tract. |
| LPCB of the coverslip from banana peel culture | LPCB stain revealed the same morphology of Aspergillus nidulans, along with fruiting body or cleistothecium showing emerging Hülle cells. |


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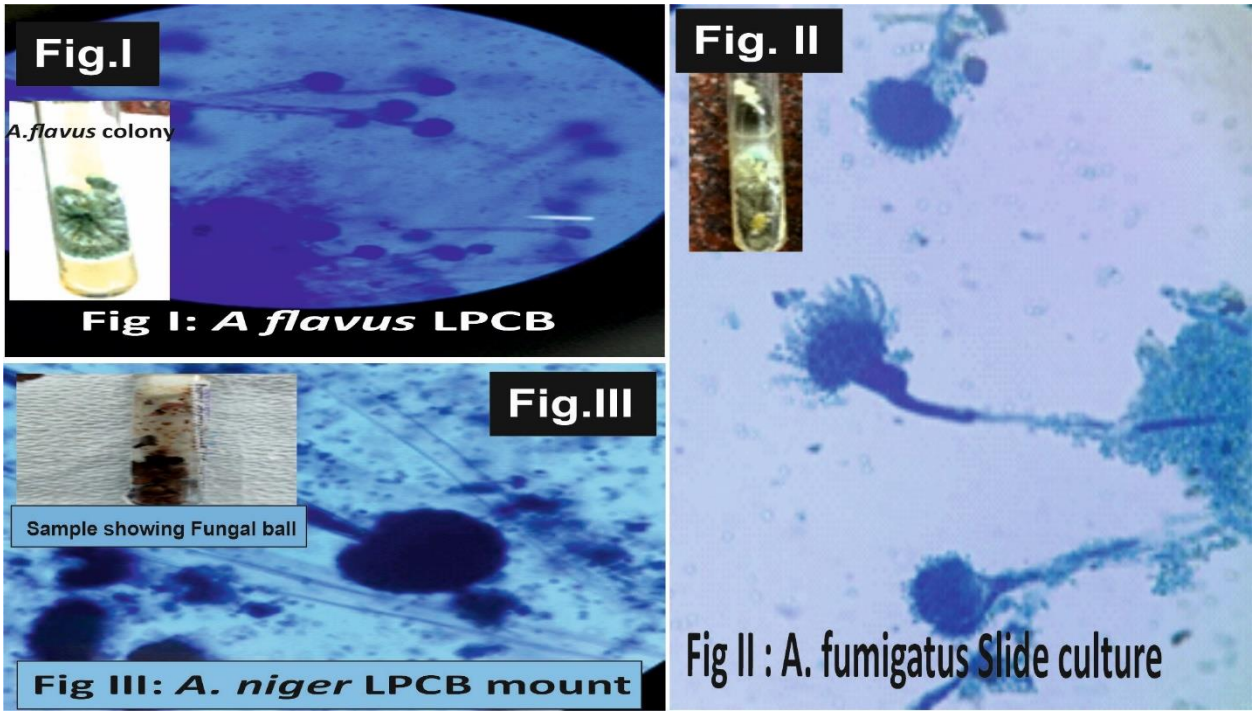
**Figure(s) I , II & III : showing A. flavus , A. fumigatus & A. niger respectively**




**Figures IV, V & VI showing A.nidulans, A. amstelodami and A. terreus respectively**

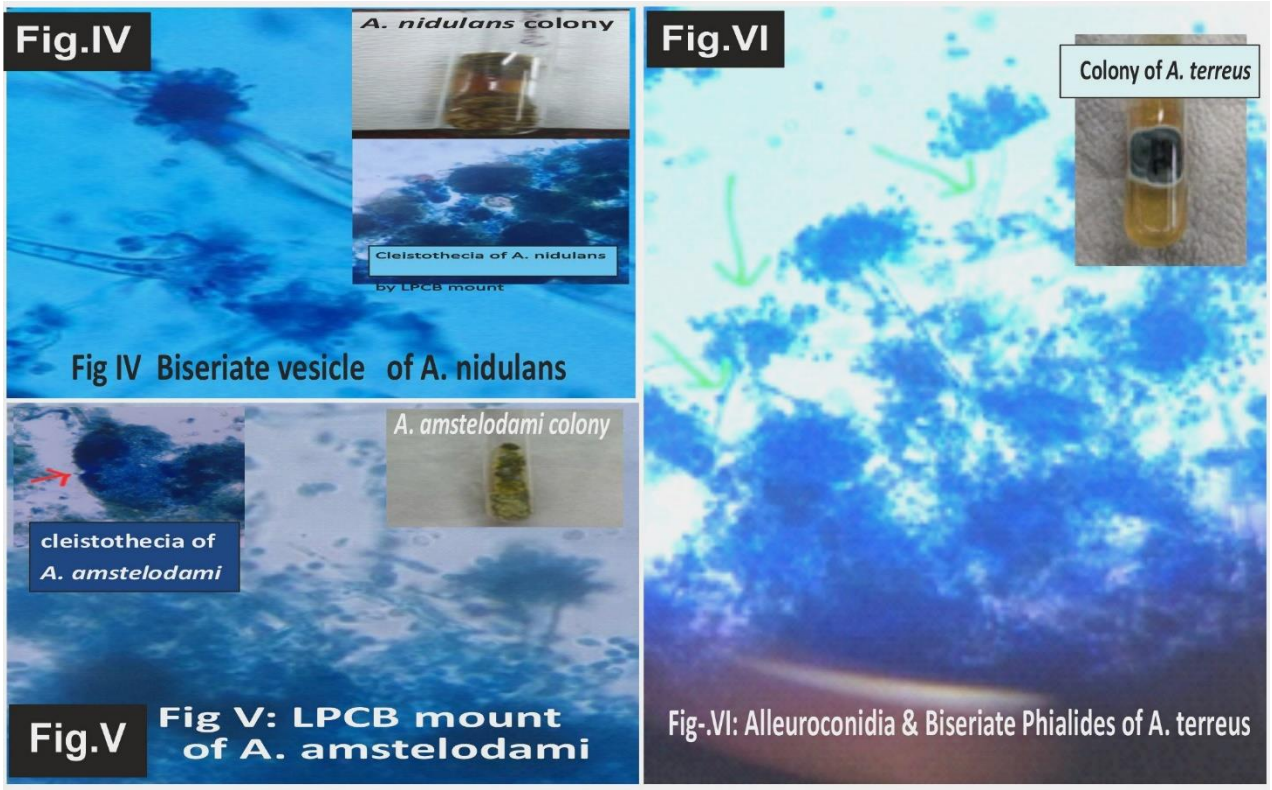




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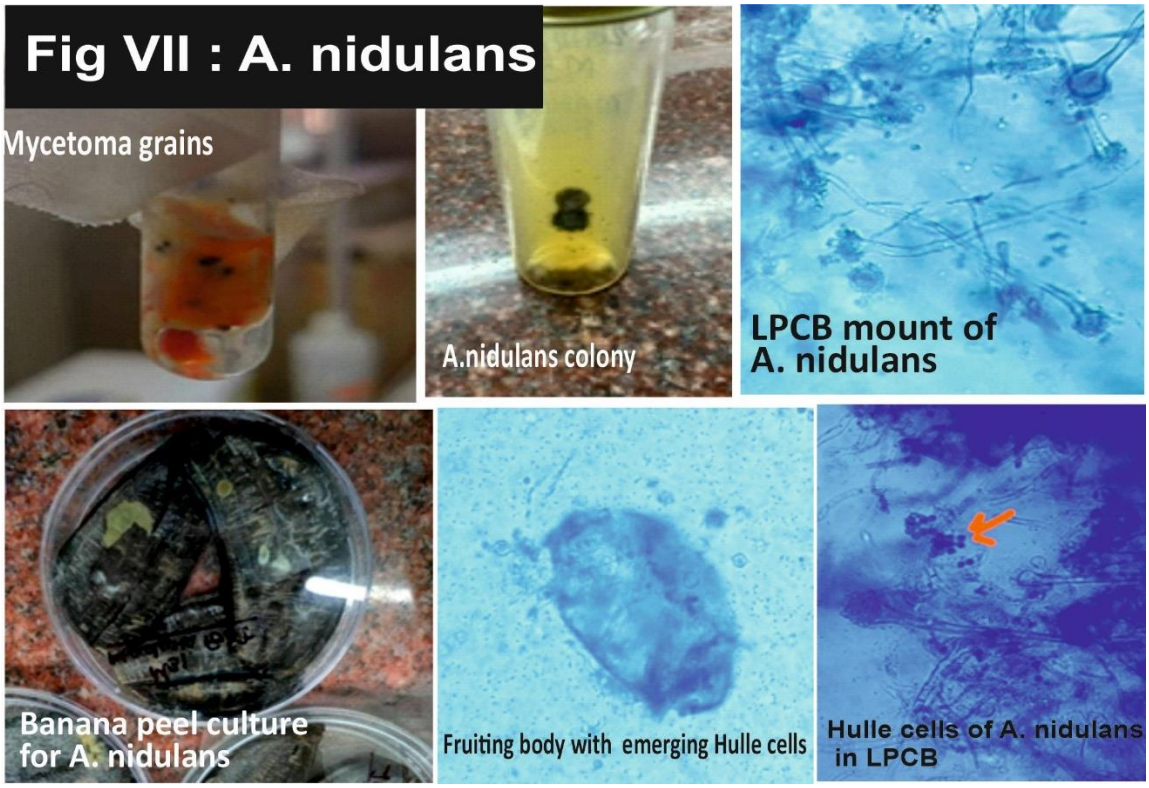
**Figure VII Showing Different Stages of Processing Black Grain Mycetoma and Morphology and Cultural Characteristics of Aspergillus nidlans**




## DISCUSSION

Here incidence of hand nail involvement is more frequent than toenails. Typically, there is early involvement of lamina and painful peritonitis without pus. Unlike dermatophytes, non-dermatophyte moulds (*Aspergillus spp*.) are usually not keratolytic. Therefore, previous keratin destruction by dermatophyte, trauma or another nail disease can favour the invasion of the nail bed by Aspergillus spp. as a secondary invader [7].

*Aspergillus spp.* are insensitive to terbinafine or itraconazole, which are usually applied for dermatophytes. A Standard treatment for onychomysosis due to *Aspergillus sp.* has not yet been established & perfect cure is rather difficult to obtain.

A review of 42 epidemiological studies due to *Aspergillus sp.* varies between <1% & 35% of all cases of onychomycosis in the general population. It is very uncommon among children & adolescents.

Aspergillosis in the urinary tract is a rare disease. In most cases invasive aspergillosis in the urinary tract is due to nosocomial infection; diagnosed in immunocompromised patients such as organ transplant, hematologic malignancies & immune-suppressive therapies, following instrumentation.

**Discussion on *Aspergillus nidulans*, the causative agent of Eumycetoma (Madura foot):**

**Clinical discussion:** Mycetoma is a slowly progressive, chronic subcutaneous granulomatous infection of skin & subcutaneous tissue clinically presented tumefaction & oozing granules from multiple discharging sinuses. It develops after traumatic inoculation with soil contaminated by causative agents, mostly affecting the lower extremities & exposed areas over the hands. The disease is indolent & disfiguring and may involve adjacent fascia & bony structures after a long duration. Complications include fractures of infected bone & bacterial superinfection. Skin shows a wooden fibrotic Induration due to dermal sclerosis, draining mycetomas are often superinfected by *Staphylococcus sp.* &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*streptococcus sp*. Patients are otherwise in good health with no satellite adenopathy.

**Etiological Discussion:** Unlike the usual fungal agents of black grain eumycetoma [8], here the isolate has been diagnosed as *Aspergillus nidulans* ( also called *Emericella nidulans*). The isolate is a homothallic fungus i,e able to self-fertilize & form fruiting bodies. Grains represent the aggregates of fungal hyphae, 2-6 µm in diameter in eumycetoma.

**Incidence & Epidemiological Discussion:** mycetoma is endemic in tropical & subtropical countries between latitudes $15^0$ N & $30^0$ S; known as the Mycetoma belt. The highest incidence is found in Sudan, Venezuela, Mexico & India. The first attempt to map the distribution of mycetoma globally was made by Abbott (1956) by studying 1321 cases of mycetoma in Sudan[9]. Sudan is considered the most endemic country & 70% of cases of mycetoma (among > 7000 patients) have been identified due to *Madurella mycetomatis* in its capital, Khartoum [10].

Extreme climatic conditions in endemic areas contributes to survival of causative agents, more commonly affect young men aged 20-40 yrs. Male:Female ratio of mycetoma cases is 3.5 : 1.

In Southeast Asia, India, and Pakistan the ratio of Eumycetoma & Actinomycetoma is 35% & 65% respectively. In India, most cases of mycetoma are found in Tamil Nadu (Madurai) and the moist southern part of Rajasthan. In India Mycetoma due to *A. nidulans* is rare, with only a few case reports of white-grain eumycetoma by this isolate [11,12].

**Laboratory Discussion:** Isolation of black grains from the tissue sample & 20% KOH mount of saline-washed crushed grains remains the crucial step. Actinomycetoma was excluded by modified ZN staining (negative). The study of granule colour, texture, size & presence of hyaline/pigmented hyphae helps determine causative agents (here it is hyaline hyphae). Isolation is slowly growing in culture, so meticulous daily observation of cultural media is needed.

LPCB staining from culture revealed the characteristic structures of *Aspergillus nidulans*. Hülle cells are thick-walled globose cells in shape, emerge from cleistothecium, and are specialized for versatile dispersion & re-genesis. These cells can grow into hyphae that develop into spore-producing colonies. During sexual development these multinucleate cells can inherit nuclei from both parents, indicating that they may serve as genetic backups.

Instead of conventional slide culture by cornmeal agar block; banana peel culture method used for easy identification of late-sporulating human fungal pathogens [6].

Banana (*Musa sapientum*) peels are high quality & cheap source of carbohydrates, & minerals & it promotes growth of reproductive structures. LPCB staining from Banana peel culture showed the same entity like a slide culture, producing fruiting bodies, which is characteristic for *Aspergillus nidulans*.

Several studies on Aspergillosis have been conducted. In one study Felix Bongomin et al. [13] studied 42 cases of onychomycosis & prevalence of aspergillosis was 7.7% of the proportion of NDMO. *A. flavus, A. terreus, & A. niger* were the most common etiological sp. Another epidemiological analysis of onychomycosis was conducted at Montpellier University Hospital, France (1991-2019) by Laetitia Laroche et al. [14] revealing nail aspergillosis prevalence of 16.9 % of cases. Analysis of clinical reports of 102 patients with ungual aspergillosis (men/women ratio: 0.67; mean age 56.3 years) identified cardiovascular, endocrine, cancer & skin diseases as contributing factors.

Though commonest species implicated in invasive aspergillosis is *Aspergillus fumigatus*, but in developing countries *A. flavus* has been isolated comparatively at higher frequency from sino-orbital Aspergillosis [15], which is closely corroborated with our study revealing Aspergillus flavus as most prevalent pathogen causing invasive Aspergillosis.

## CONCLUSION

It is nearly impossible to avoid exposure to *Aspergillus sp*. *A. flavus* & some other isolates can produce aflatoxin (mycotoxin), having carcinogenic potential. Environmental stress can upregulate aflatoxin production by the fungus, which can occur when the fungus is growing on damaged plants. Galactomannan (GM), a major component of the Aspergillus cell wall; is released during invasive disease. The level of circulating serum or plasma GM is indicative of fungal burden in the host. A positive GM result should be considered along with microbiologic


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culture, histology of biopsy specimen & radiographic evidence. The use of GM EIA & qPCR assay in conjunction with culture-based diagnostic methods applied to BAL fluid could facilitate accurate diagnosis & more timely initiation of specific therapy. Posaconazole, an extended-spectrum Triazole antifungal agent is effective against *Aspergillus*.

The global emergence of azole-resistant *A. fumigatus* isolates notoriously limits the therapeutic options. Although specific mutations in CYP51A are the main cause of azole resistance, there is a new wave of azole-resistant isolates with wild-type CYP51A genotype challenging the efficacy of the current diagnostic tools. Therefore , applications of whole genome sequencing are increasingly gaining popularity to overcome such challenges.

Diagnosis of invasive aspergillosis remains difficult in that clinical manifestations are not specific; radiologic findings can be suggestive but not pathognomonic. Along with definite LPCB morphology from fungal growth, histologic demonstration of invasive hyphae represents proven invasive fungal diseases .

**Ethical approval:**

The study was conducted after meeting all ethical considerations as per the declaration of Helsinki.

**Conflict of Interest:** There is no conflict of interest involved in this study.

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**Availability of data and materials**

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## HIGHLIGHTS

1. This original research article represent a prospective study to determine the prevalence of Aspergillosis in superficial, systemic & subcutaneous infections.
2. In this study diagnosis & speciation of *Aspergillus* isolates has been done to ascertain the significance of specific fungal isolate in causing local and systemic pathogenesis.
3. This study has also revealed *Aspergillus nidulans* as a causative agent of Black Grain Eumycetoma in Indian sub=continent.

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