**Original article**

# Antibody response in central nervous system to the antigenic preparation of *Mucor* and *Aspergillus*

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## Abstract

**Introduction and objective:** *Aspergillus* and *Mucor* are found to cause life threatening infections. Though cerebral aspergillosis and mucormycosis are rare, the disease mortality rate is found to be predominant in patients with immune system being compromised. Because of the high mortality rate of this infection, early diagnosis and prompt initiation of treatment are crucial as effective treatment improves the overall management of diseases. The objective of the present study was to screen for a method in diagnosing cerebral aspergillosis and cerebralmucor-mycosis.

**Materials and methods:** In the present study the antigens were effectively extracted from *Aspergillus* and *Mucor,* and then they were characterized using native polyacrylamide gel electrophoresis, sodium dodecyl sulfate polyacrylamide gel electrophoresis, and their diagnostic potential was evaluated by ELISA.

**Results:** Among the bands visualized *Aspergillus flavus* showed four distinct bands, two bands in *A. fumigatus* but a diffused band was noticed with *Mucor*. Clinical analysis by ELISA conducted in 47 cerebro spinal fluid samples from patients suspected for chronic meningitis infection were found to be positive for antigens in 8.51%, 6.38% and 12.76% for *A. flavus*, *A. fumigatus* and *Mucor*, respectively.

**Conclusion:** From the results, it was concluded that ELISA can be used as a sensitive and reproducible test for diagnosis of cerebral aspergillosis and mucormycosis.

**Significance and impact of the study:** With regard to the study on the fungal antigen which infects the central nervous system and also understanding the importance of ELISA as a sensitive and reproducible diagnostic tool, early diagnosis of cerebral aspergillosis and mucormycosis is possible.

**Keywords:** Cerebral aspergillosis; Chronic Meningitis infection; ELISA; Mucormycosis

## Introduction

Central nervous system (CNS) aspergillosis is an opportunistic infection that mainly affects immunocompromised individuals, such as AIDS patients, transplant recipients, patients with haematological diseases, or patients with autoimmune diseases treated with immunosuppressive regimen [1]. Since the diagnosis is difficult and the antifungal therapy is often with poor efficacy, the mortality rate is very high. Lethality of CNS aspergillosis exceeds 90% and lethality of cerebral mucormycosis ranges from 21-70% [2].

Imaging techniques may be suggestive of mucormycosis but are rarely confirmative. A high index of suspicion is required to make the diagnosis of rhinocerebral mucormycosis, as evidenced by the fact that autopsy series have found up to half of the cases which are diagnosed by postmortem. Still, as with CT scans, patients with early rhinocerebral mucormycosis may have a normal magnetic resonance imaging, and surgical exploration with biopsy of the areas of suspected infection should always be performed in high-risk patients [3]. Culturing organisms from a potentially infected site is rarely sufficient to establish the diagnosis of mucormycosis because the causative agent is ubiquitous, may colonize normal individuals, and is a relatively frequent laboratory contaminant [4]. There are no reliable serologic, PCR-based, or skin tests for mucormycosis. Therefore, the diagnosis is often made by biopsy of infected tissues.

Diagnosis of cerebral mucormycosis is not simple and the demonstration of fungal elements from cytologic preparations is complicated by the difficulty in extracting fungal elements from invaded tissues [5]. Fungal elements may be rare in cytological specimens and when present are often fragmented. Additionally, hyphae may be focal and appear in only part of a specimen. A delayed diagnosis means that appropriate doses of antifungal treatment are started late, which can have the consequence of increasing the number of failures.

On the other hand diagnosis of aspergillosis is based on direct examinations and culture [6]. Establishing a diagnosis of *Aspergillus* meningitis is difficult, and many cases have been diagnosed only by autopsy [4]. Typically only a small number of fungal cells are present in cerebrospinal fluid (CSF). A positive culture may be obtained if a large volume of CSF, preferably 5ml or more, is cultured. The sudden onset of neurological symptoms in a febrile immunocompromised patient with extensive pulmonary infiltrates and multiple abscesses with mass effect on MR suggest the diagnosis of cerebral aspergillosis.

Immunoassays with *Aspergillus* antigens purified by biochemical procedures have only recently been reported [7]. A minor contamination of even, 1% of the antigen of interest with another antigen of greater reactivity may lead to erroneous results. To avoid such problems, it is now possible to use molecular biological techniques to produce pure recombinant antigens. Such antigens serve as the basis for the development of ELISA methods which will allow the quantitation of the antibody response [7].

The serologic markers of fungal infection gave false negative in many cases. First, the levels of fungi in both blood and CSF were below the lower limit of detection of conventional diagnostic assays. Second, the CNS infected with an uncommon fungal pathogen that could not be detected by conventional diagnostic assays [7]. Therefore, the benefits of PCR diagnosis and screening of blood samples is limited if sampling takes place once treatment has started. The diagnosis of CNS fungal infection can be confirmed by brain

biopsy, but an invasive diagnostic procedure is not always feasible for immunocompromised patients [8]. Since early diagnosis greatly increases a patient's chance of survival, a rapid diagnostic test is needed. Thus, this study is mainly concerned with preparing the antigen from *Aspergillus* and *Mucor* for diagnostic purposes.

## Materials and methods

*Patients and sample*

A total of 47 patients suspected for chronic meningitis infection (tuberculosis, cysticercosis, neurosyphilis, cancer and fungal infections admitted to the Neurology department of Thanjavur Medical College, Thanjavur, Tamilnadu, on whom lumbar puncture was performed and samples were collected.

*Preparation and extraction of antigens*

The preparation and extraction of antigens were followed according to the modified procedure of Kenneth Jones and Leo Kaufman [3]. The cultures of *A. flavus, A. fumigatus* and *Mucor* were taken and sub cultured on Sabouraud dextrose agar media (SDA, Fisher Scientific, India) slants separately for two weeks to get the pure cultures. After two weeks, the pure cultures were inoculated in separate sterilized flasks containing 250ml of sterilized Sabouraud dextrose broth each, and grown at room temperature on a shaker.

The fungi were allowed to grow for two weeks. After two weeks, the cultures were killed by adding 1% formalin and then subjected to UV for two and half hours with periodic mixing of the flasks contents. After 24h, the viability of the fungi was checked by inoculating it on sterilized SDA slant for 24h at room temperature. The extraction of antigen was done after ensuring the loss of viability of the fungi, by the absence of the fungal growth on the SDA slant.

After checking the viability of the fungi, the mycelial mat from the broth culture were separated using Whatman no: 1 filter paper. The mycelial cultures thus separated were filtered and taken in three different sterilized flasks. To each flask, 20ml of phosphate buffer saline, 20ml distilled water and 100µl of 1% Thiomersal was added and heated at 80°C for two hours in water bath with periodic sonication. Then the cultures were subjected to ultrasonication for 10mins at 10sec pulse. After ultrasonication, the cultures were transferred to separate centrifuge tube and ultracentrifuged at 18,000rpm for 10mins at 7°C. The supernatant is then separated into sterilized tubes. The volume of antigen extracted from these fungi, *A. flavus, A. fumigatus* and *Mucor*, was 15ml each. The antigens were concentrated to 1.5ml each, using the Speed Vac (Heto vacuum centrifuge, India).

*Characterization of the extracted antigen*

For the characterization of the antigen, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Native PAGE was performed and stained with PAGE blue and silver staining. In all cases proteins were visualized in the gels by Commassie blue R-250 staining and silver staining [9].

*Page-blue staining*

The gel was fully submerged in a container containing the Coomassie blue stain for staining. The gel was stained for 1h, and agitated slowly on a shaker. The gel was then de-stained in a de-staining solution a few times until protein bands were visualized. The molecular weight of the visualized protein bands were determined by comparing them with the molecular weight markers.

*Silver staining*
The gel was transferred into distilled water and kept for 10mins on a shaker. The gel was then fixed in fixing solution containing 50ml methanol, 12.5ml acetic acid, 37.45ml distilled water and kept on the shaker for 30min. The gel was then washed in 150ml of 50% methanol for 10mins on the shaker. The gel was then washed in distilled water for 10mins on the shaker. Now the gel was transferred into solution containing 0.005mg of sodium thiosulphate in 25ml distilled water for 1min on the shaker.

The gel was then washed in distilled water for 1min on a shaker. Solution containing 0.025mg silver nitrate, 25ml distilled water and 15µl of formalin was prepared. The gel was kept in this solution for staining for 20mins in dark condition. The gel was then washed twice in distilled water for one minute each on a shaker. Solution was made by adding 0.5g sodium carbonate and 15µl of formalin to 25ml distilled water. The gel was transferred into container containing this solution and shaken gently till the color developed. Once the color developed, the gel was transferred into container containing 2.5ml acetic acid and 50ml distilled water to stop the reaction.

*ELISA*
In the present study indirect ELISA was performed by modified method of Rajpal Kashyap *et al.* [5]. ELISA plates were first coated with the fungi antigens (50µl per well) were mixed with PBS, and kept overnight at 4°C. Subsequently, the plates were washed thoroughly with PBS-T and tapped dry. 120µl of 1% milk in PBS-T was added in each well (Anikspray, India) and incubated at 37°C for two hours. The contents of the plates were discarded and tapped dry. The CSF samples were collected from patients suspected for chronic meningitis infection including tuberculosis, cysticercosis, neurosyphilis, cancer and fungal infections.

Dilutions were prepared by mixing 450µl of milk and 50µl of samples (CSF) in different dilution tubes. 50µl duplicates were added in each well. The plates were then incubated at 37°C for two hours. At the end of incubation, plates were washed five times with PBS-T, tapped dry and 50µl of secondary antibody was added to each well. These plates were then incubated at 37°C for one and half hours. At the end of incubation, the plates were washed five times with PBS-T. 75µl of O-Phenylene Diamine was added to each well and kept in dark till the colour develops. Then the reaction is arrested by adding 50µl of dilute sulpuric acid. Optical densities of the wells' contents were read at 492nm using ELISA reader.

## Results
The antigens were characterized by SDS PAGE, Native PAGE which was subjected to PAGE blue and silver staining. The proteins were visualized in the gels by Commassie blue R-250 staining and silver staining (Fig. 1). In this study, *Aspergillus flavus* showed proteins which were eluted at a region of 47kDa, 33 kDa, 24kDa and 20kDa. *A. fumigatus* showed peptides recovered roughly in the region of 79kDa and 47kDa. The results were found to be positive for antigens in this present investigation for ELISA which were as follows: 8.51% (4 out of 47 samples) for *A. flavus*, 6.38% (3 out of 47 samples) for *A. fumigatus* and 12.76% (6 out of 47 samples) for *Mucor* of the CSF samples collected from patients suspected for chronic meningitis infection (tuberculosis, cysticercosis, neurosyphilis, cancer and fungal infections) (Fig. 2).

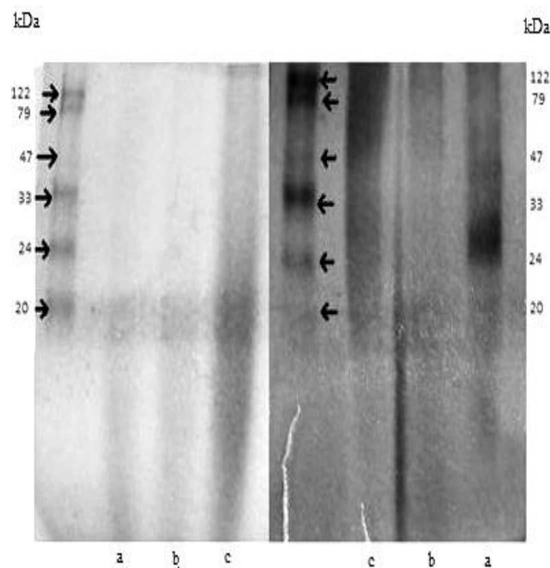


**Fig. 1:** Image depicting page blue staining and silver staining of the antigens (a) *A. flavus*, (b) *A. fumigatus* and (c) *Mucor*

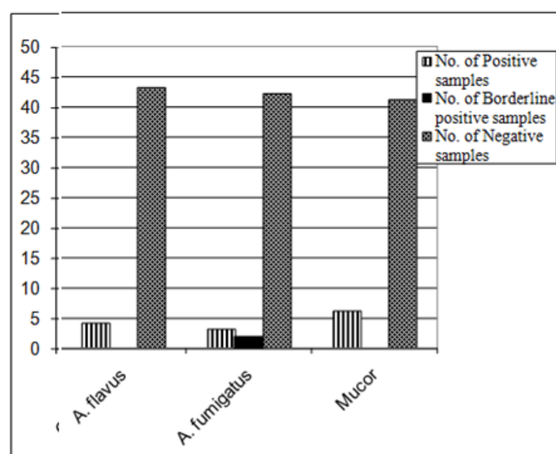


**Fig. 2:** Histogram showing percentage of CSF antibody positivity to antigens of *A. flavus, A. fumigatus* and *Mucor*

## Discussion

*Aspergillus* and *Mucor* cause disease in various organs including the CNS resulting in cerebral aspergillosis and mucormycosis which are rare but highly fatal diseases and hence a need for diagnosis at an early stage is extremely crucial. When 15ml antigens of *Aspergillus* and *Mucor* extracted from CSF sample of Tanjavur Medical College patients who were subjected to PAGE, a series of protein bands were visualized. The findings were similar to the results obtained by other researchers [10,11]. In this study *A. flavus* showed proteins which were eluted at a region of 47kDa, 33kDa, 24kDa and 20kDa, *A. fumigatus* showed peptides recovered roughly in the region of 79kDa, 47kDa, where as the antigens extracted by Latge *et al*. [5] had molecular mass of 18 to 20kDa (Fig.1).

The native and SDS PAGE protein profile of *Mucor* did not reveal distinct band but a diffused band was noticed in the respective lane. Further characterization of the antigen and evaluation of the antibody reactivity in CNS infections was done by ELISA. ELISA is a simple and rapid method for diagnosis of cerebral aspergillosis and mucormycosis. Apart from being sensitive and reproducible, previous studies made by many scientists revealed that the test is reliable even when used on bronchoalveolar lavage or CSF [12].

In our study 8.51% (4 out of 47 samples), 6.38% (3 out of 47 samples) and 12.76% (6 out of 47 samples) of the CSF samples collected from patients suspected for chronic meningitis infection (tuberculosis, cysticercosis, neurosyphilis, cancer and fungal infections), showed positive results by ELISA for antigens of *A. flavus, A. fumigatus* and *Mucor*, respectively (Fig. 2). Whereas, in the study by Latge *et al.* [5] for the diagnosis of the aspergilloma and invasive aspergillosis, urine samples were used and comparatively larger number of samples were positive. Not much study is made on *Mucor* antigen and its characterization for diagnostic tools.

Infections of CNS with fungi can cause devasting consequences. Several fungi may cause infection in normal humans, most of them are opportunistic and influence immunocompromised host [13] thus, study

proves rapid ELISA can be used in diagnosis which is crucial to treatment.

## Conclusion

Rapid ELISA is simple to perform and appears to be a suitable method for routine application in the serological diagnosis of cerebral aspergillosis, but further studies might be necessary in case of cerebral mucormycosis. This study will be useful for early diagnosis of cerebral aspergillosis and mucormycosis.

**Conflict of interest statement:** All authors declare that they have no conflict of interest.

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