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Evaluation of Five Methods for Preparation of *Aspergillus* Antigen for the Diagnosis of *Aspergillus* Infections

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Abstract

Background: Aspergilli are saprophytic fungi, which cause a wide spectrum of diseases. Invasive aspergillosis is an increasing infection in immuno-compromised patients, especially in patients with chronic diseases such as diabetes, alcoholism, patients taking steroids, organ transplant patients, cystic fibrosis, AIDS, chronic obstructive pulmonary disease, chronic granulomatous disease, inactive tuberculosis, and severe asthma[1].

Objective: To evaluate five methods used for the preparation of *Aspergillus* antigen for the diagnosis of *Aspergillus* infections

Materials and methods: **A** sensitive and specific antigen was prepared from cells of *Aspergillus flavus* and *Aspergillus fumigates* that cause infection of the nasal sinuses and lungs in humans. Five methods were chosen for *Aspergillus* cell wall rupture to release the antigen. Two mechanical methods: one performed by an ultrasonic machine and the other by vortex mixture of glass beads. A physical method was used for freezing and thawing with liquid nitrogen and water bath. A chemical method using toluene and a manually crude method by pestle and mortar were also used. The antigen was tested by the counterimmunoelectrophoresis(CIE) machine against 209 aspergillosis patients' sera: 152 sera test for *Aspergillus flavus* (*A. flavus*) antigen, and 57 sera for *Aspergillus fumigates* (*A. fumigatus*) antigen.

Results: The physical and the two mechanical methods released a high level of antigen and gave strong positive bands in agar gel against the antibody in patients' sera. While the crude method gave strong and faint positive bands. The chemical method antigen with its lower concentration gave faint positive bands.

Conclusion: The mechanical ultrasonic, mechanical glass beads, and physical methods yielded the strongest positive bands. The counterimmunoelectrophoresis technique is important for early diagnosis and follow- up of nasal sinusitis and pulmonary aspergillosis.

Keywords: *A. flavus*, *A. fumigatus*, Counterimmunoelectrophoresis, Ultrasonic machine.

Introduction

There are a high mortality from aspergillosis. *A. fumigatus* and *A. flavus* are the most frequent infections of systemic aspergillosis. *Aspergillus fumigatus* was the most commonly isolated in pulmonary aspergilloma. *Aspergillus flavus* was the most commonly isolated in paranasal sinuses aspergilloma[2].

The clinical diagnosis of invasive aspergillosis is often difficult, since the signs and symptoms of infection are nonspecific. Paranasal sinuses mycoses is common in northern India, northern Sudan, and south-western states of USA. Different techniques were used to prepare the antigen which helps in serology testing for rapid diagnosis of invasive aspergillosis. That preparation of antigen depends on culture of *Aspergillus* cells[3,]

Five techniques were used to disintegrate the cell wall depending on the adherence protocols of cell wall rupture of fungal strains by grinding the separate mycelium mat manually in a pestle and mortar, mycelium homogenization with glass beads milling on vortex mixture, crackle under liquid nitrogen, and ultrasound disintegrating by an ultrasonic machine.· An alkaline buffer solvent was applied to extract the cytoplasm. Sera of patients were used to test for presence of *Aspergillus* antigen[4].

CIE in agar gel was used as a serological test. It depends on the binding and precipitating an antibody to a specific antigen. Mycological and serological studies for paranasal *Aspergillus* granuloma and for bronchopulmonary aspergillosis were prevalent in Sudan and caused by *Aspergillus flavus* and *Aspergillus fumigatus*. *Aspergillus* granuloma is found mainly in Sudan and some tropical countries, and it involves the paranasal sinuses orbit and the brain[5].

The serological test depends on the antigen preparation which is important to help quick diagnosis to detect the disease rapidly, to facilitate the antifungal treatment, and to follow-up *Aspergillus* patients. This work reviewed the recent advances in non-culture methods for the diagnosis of invasive aspergillosis.

Materials and methods

The study was an analytical case study conducted in the Mycology Reference Laboratory, Department of Microbiology, Faculty Medicine, University of Khartoum (Khartoum State, Sudan). The study was approved by the Scientific Research Committee of University of Khartoum. Verbal informed consent was obtained from all patients. Data confidentially was maintained, and the specimens and information collected from all participants had not been used for any purpose other than this study.

A total of 209 human sera (152 sera of nasal sinusitis and 57 sera of pulmonary infection) were investigated. *Aspergillus* strains were isolated from nasal biopsy and sputum by agar culture, and identified morphologically to be typical *A. flavus*, strain BT2b (Lab. No. 415) and *A. fumigatus*, strain CAL (Lab. No. 413). Isolation was performed by culture on Sabouraud's dextrose chloramphenicol selective agar medium. These two strains were used to prepare *Aspergillus* antigens. 5ml venous blood were collected from patients infected with *Aspergillus.* The culture medium used for preparation of antigen was Sabouraud's dextrose broth that consists of: peptone (10 grams), dextrose (20 grams), distilled water (1000 ml), chloramphenicol (0.05 gram), and final pH 5.4.

Culture methods: *Aspergillus* species colonies were transferred into flasks of 500 ml containing 250 ml Sabouraud's dextrose broth with chloramphenicol (SDA+Ch) and incubated at 26°C for 4 weeks. 10 ml of 1% formalin was added to *Aspergillus* growth and incubated for 24 hours to kill the growth. Fungal mycelia were harvested from the broth using Whatman No. 1 filter paper. Then phosphate-buffer saline (PBS) fluid was added to the harvest to make the fungal cells ready

for antigen production. The suspension was centrifuged at 6000 rpm for 20 minutes. Dialysis of the suspension was performed in a dialysis bag and the concentrated antigen so formed was frozen at (- 80°C) until further analyzed.

Antigen preparation methods: The following five methods were applied:

1. **Mechanical ultrasonic method**: A sonicater machine was used to rupture of the fungal cell wall. 10 g of the separated mycelia were matted in 20 ml phosphate-buffer saline fluid (pH 7.4). The mixture was placed in a 125 ml flask and the suspension was kept cool by placing the flask in a beaker containing ice cubes, then transferred to a 60-W MSK ultrasonic disintegrator for 20 min. The suspension was centrifuged at 6000 rpm for 20 minutes in a cool place. The supernatant was transferred to dialysis bags. The dialysis bags were placed in a flask containing distilled water to dialyze the antigen at 4° C for 24 hours. The dialysis bags were covered by a coarse polyethylene glycol 6000 powder to concentrate antigen. The resultant concentrated antigen was stored at (-80°C) until later use.

2**. Mechanical glass beads method**: This was performed by a vortex mixture machine. First the glass beads (diameter 4.5 mm) were washed with 80 ml acetic acid. Then 20 g of the wet mycelia, 30 ml phosphate-buffer saline, and the glass beads were mixed in a 250 ml flask (pH 7.4), placed in the vortex mixture machine for 15 min at 4°C and at the maximum speed. After that the glass beads and cell debris were deposited by centrifugation at 6000 rpm for 20 minutes at 4°C. The supernatant was transferred to dialysis bags and distilled water was poured into the flask to dialyze the antigen, and kept at 4°C for 24 hours. Dialysis bag was covered by polyethylene glycol 6000 to concentrate antigen. The resultant antigen was stored at (-80°C) until later use.

3. **Physical method**: The physical method for rupture of the fungal cell wall depends on freezing with liquid nitrogen at (-196°C) and thawing in a water bath at 80°C. *Aspergillus* cells were grinded for several times under liquid nitrogen. Then the mycelia were placed in a sterile container into the liquid nitrogen cylinder for 5 minutes. The frozen mycelia were then put into a water bath for 5 minutes. Phosphate buffer saline (pH 7.4) was added and the mixture was grinded with mortar and pestle, and centrifuged at 6000 rpm for 20 minutes. The supernatant was transferred to a dialysis bag, distilled water was added, and the flask was kept at 4°C for 24 hours. The dialysis bag was covered by polyethylene glycol 6000 to concentrate antigen. The resultant antigen was stored at (-80°C) until later use.

4. **Chemical method:** A chemical solvent was use to excrete the cytoplasm from the fungal cells. We use toluene at 100% concentration in an alkaline buffer (pH 8.0) to rupture the fungal cell wall in 3 days. The mixture was centrifuged at 6000 rpm for 20 minutes. The supernatant was poured in dialysis bags and were put in a flask containing distilled water to dialyze the antigen, and the product was kept at 4°C for 24 hours. Dialysis bags were covered by polyethylene glycol 6000 to concentrate the antigen. The resultant antigen was stored at (-80°C) until later use.

5. **Crude method:** The filtered mycelia were torn by pestle and mortar and phosphate-buffer saline was added. The fugal cells were continually crushed until a soup-like suspension was formed. This suspension was centrifuged at 6000 rpm for 20 minutes at 4°C. The supernatant was transferred to dialysis bags inside a flask containing distilled water and kept at 4°C for 24 hrs to dialyze the antigen. Dialysis bags were then covered by polyethylene glycol 6000 to

concentrate the antigen. The resultant antigen was stored at (-80°C) later use.

Serological Test: Here the CIE technique was used (Gumaa, *et al*,1972)[6]. The test was performed in 1.5% purified agar gel and 0.9% sodium chloride with a buffer created from 2.7grams sodium barbitone, and 2.2 grams sodium acetate at pH 8.6. The glass slides were placed on the chamber surface, and coated with 100 ml agar. After pouring the gel the mixture was allowed to set for 10 minutes, to give a thickness. The gel was punctured by a 3 mm diameter borer. The inter-well spaces in each column were 2 mm, and leaving a 2 cm margin. 2 µl *Aspergillus* suspension (antigen) and the serum samples (antibody) were added to the respective well with micropipette tips. The standard positive sera were regularly employed as a control. Then, the moist chamber was transferred to a Shandon Vokam power pack apparatus and buffer was poured inside the tank. Charge pattern of the antibody wells was made towards the anode, and the antigen wells were connected to the cathode. This was kept at 4ºC for 2 hours. Then the moist chamber was transferred to the washing buffer container (sodium chloride 4 grams, sodium tetraborate 4 grams, and distilled water 1000 ml) and kept overnight. The slides were separated from each other and dried with filter paper.

The final stage was to stain the slides by covering them with 0.5g Naphthalene black, 500 ml methanol, 100 ml glacial acetic acid and 400 ml distilled water, and followed by immersing the stained slides in a differential solution. All was left to dry and examined under oblique illumination for immuno-precipitate between the wells of serum (antibody) and the antigen.

Results

10 *Aspergillus* antigens were prepared by the mechanical ultrasonic method, the mechanical glass beads method, the physical method, the chemical method, and the crude method. Five antigens were prepared from each of *Aspergillus flavus* and *Aspergillus fumigatus.* All five procedures had yielded high concentrations of *Aspergillus* antigens.

By the sonication technique, the antigen of *A .flavus* yielded strong positive bands with 43 patients' sera, and 109 sera were negative; while the antigen of *A. fumigatus* yielded strong positive bands with 6 patients' sera, and 51 sera were negative.

By the bead mill technique, *A. flavus* antigen yielded faint positive bands with one patient's serum and 109 sera were negative; while the antigen of *A. fumigatus* yielded strong positive bands with 6 patients' sera, and 51 sera were negative.

By the freeze and thaw technique, the antigen of *A. flavus* yielded strong positive bands with 43 patients' sera, and 109 sera were negative; while the antigen of *A. fumigatus* yilded strong positive bands with 6 patients' sera, and 51 sera were negative.

By the crude technique, the antigen of *A .flavus* yielded strong positive bands with 37 patients' sera, faint positive bands with 6 patients' sera, and 109 sera were negative; the antigen of *A. fumigatus* yielded strong positive bands with 3 patients' sera, faint positive bands with 3 patients; sera, and 51 were negative.

By the chemical technique, the antigen of *A..flavus* yielded strong positive bands with 21 patients' sera, faint positive bands with 22 patients' sera, and 109 sera were negative; while the antigen of *A. fumigatus* yielded strong positive bands with 4 patients' sera, faint positive bands with 2 patients' sera, and 51 sera were negative.

Descriptive statistis was carried out by the SPSS program.
Fig. (1) shows the reactions of *A. flavus* antigen against the sera antibodies using the five methods. 72% of the overall sera investigated yielded negative result. The sonicater, glass beads, and freeze thaw techniques showed 28% strong positive bands, and 0% faint positive bands. While the crude method yielded 26% strong positive bands, and 3% faint positive bands. The toluene method showed 11% strong positive bands and 17% faint positive bands.

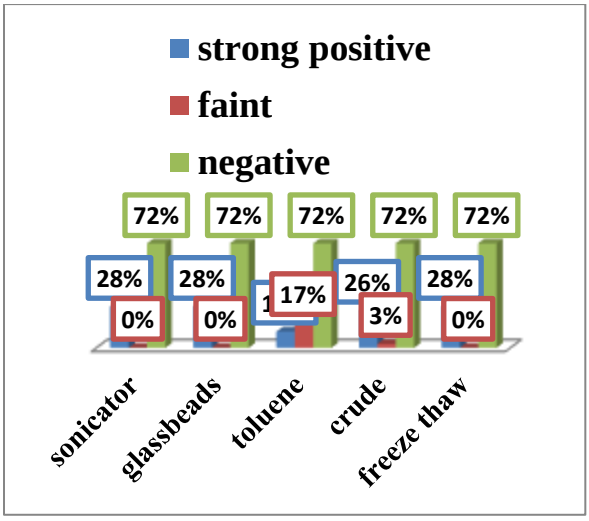

Fig (1): Reactions of *A. flavus* antigen against sera antibodies

Fig. (2) showed the reactions of *A. fumigatus* antigen against the sera antibodies using the five methods. Also, 72% of the overall sera investigated yielded negative result. The sonicater, glass beads and freeze thaw techniques showed 11% strong positive bands and 0% faint positive bands. While the crude method yielded 5% strong positive bands, and 5% faint positive bands. The toluene method showed 7% strong positive bands and 4% faint positive bands.
The mechanical ultrasonic, mechanical glass beads, and physical methods yielded the strongest positive bands. The crude and manual methods produced a moderate amount of antigen; while the toluene method produced the least amount of antigen.

Discussion

Aspergillus is a species of saprophytic fungi found in environmental sources, including foodstuffs, soil and plants. Presence of pathogenic fungi must be carefully examined, along with signs and symptoms as well as medical history. Being agents of opportunistic infections, *Aspergillus flavus*, and *Aspergillus fumigatus* are the most common agent in causation of aspergillosis. An antigen is a foreign substance that induces an immune response in the body, especially in the production of antibodies. The serological diagnosis of invasive aspergillosis based on the detection of specific antigens and antibodies are used to determine if an individual has, or recently had, a specific fungal infection as it is more rapid than fungal cultures. In this study *A. flavus* and *A. fumigatus* cell wall disruption techniques, including ultrasound disintegration, homogenization with bead mill, appeared to lead to obtaining free extracts

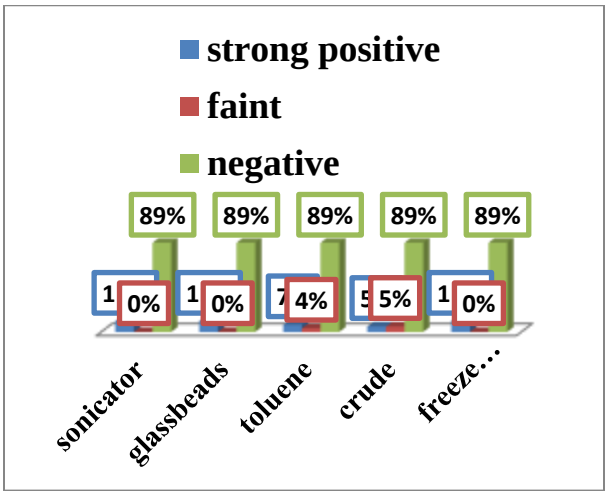

Fig (2): Reactions of *A. fumigatus* antigen against sera antibodies

containing high concentrations of soluble proteins. This finding agrees with that reported by Mahgoub (1971)[7], Ochab *et al* (2011)[8], and Hao Wei *et al* (2008)[9]. The physical method which uses freezing and thawing to rupture the *Aspergillus* cell wall with liquid nitrogen to excrete the antigen, and a high concentration of the antigen was released. This finding agrees with Margaritis (2007)[10] and Taskovaa, *et al*[11].

The crude method used a manual technique by a pastel and morter, that yielded a low concentration of antigen. This finding agrees with Kurup (2005)[12], Yeo, *et al* (2001)[13].

The chemical solvent method which uses toluene, yielded the lowest concentration of the antigen. This finding agrees with the report of Margaritis (2007)[10].

CIE was a serology test used in the diagnosis of *A. flavus* and *A. fumigatus* antigens against antibodies in patient's sera. This agrees with that reported by Hope, *et al* (2005)[14] and Weig *et al* (2001)[15]. This study was intended to evaluate and compare five antigens which were prepared from *A. flavus* and *A. fumigatus* cells, varying in their sensitivity and specificity.

Conclusion: The mechanical ultrasonic, mechanical glass beads, and physical methods yielded the strongest positive bands. The counterimmunoelectrophoresis technique is important for early diagnosis and follow- up of nasal sinusitis and pulmonary aspergillosis.

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