Advances in Life Science and Technology
ISSN 2224-7181 (Paper) ISSN 2225-062X (Online)
Vol.34, 2015

SYSTEMIC PHAEOHYPHOMYCOSIS DUE BY *ALTERNARIA ALTERNATA*: CASE REPORTS, IN-VITRO SENSITIVITY AND EXOANTIGEN STUDIES.

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ABSTRACT
Alternaria alternata is the most frequently encountered species of *Alternaria* causing opportunistic mycoses. We report two cases of phaeohyphomycosis in which *Alternaria alternata* was isolated from peripheral blood samples of 35 year and 21 year old male TB and post operative patients respectively. The direct microscopic examination of the blood samples revealed dark dematiaceous, septate mycelium. *A. alternata* was repeatedly isolated from the clinical samples of both the patients. Ketoconazole with the MIC of 10 μgml^{-1} was found to be the best effective drug against both the *Alternaria alternata* strains tested in vitro. In the present study we tried to evaluate the role of exoantigens in the serological diagnosis of such infections. The exoantigen of *A. alternate* on SDS-PAGE analysis exhibited two bands of 15Kda and 67Kda. The antisera raised in rabbit against. *A alternata* exoantigen showed humoral response after one week of immunization of ODD method. No cross reactivity was seen with antisera raised in rabbit against *Curvularia verreculosa and Cladosporum cladosporiodides.* Formation of specific precipitin bands and no cross reactivity suggests the usefulness of *Alternaria alternata* exoantigen as diagnostic tool. The antisera analysis by SDS-PAGE revealed several additional bands particularly higher than 67 Kda molecular weight as compared to the normal (unchallenged) sera from the same rabbit. Whether any of these bands could be treated as specific marker for diagnosis needs further investigations.
Keywords- opportunistic, phaeohypomycosis, *Alternaria alternata*, minimal inhibitory concentration (MIC)

INTRODUCTION

In 1974 Ajello et al proposed the term Phaeohyphomycosis to cover all infections of cutaneous, subcutaneous and systemic nature. It causes a wide range of disease including phaeohyphomycosis, chromoblastomycosis and eumycotic mycetoma (Brandt el al. 2003). Phaeohyphomycosis is a clinical entity caused by dematiaceous fungi. *Alternaria alternata* is a phaeoid fungi commonly found in man's environment and has been the most frequent species involved in human cases with severe underlying disease or in those receiving immunosuppressive drugs (Di silvero et al. 1986, Galgoczy. et al. 1985, Hernaz et al. 1983, Stenderup et al. 1987). In the present paper we report two cases of *Alternaria alternata* infection where it has been isolated from peripheral blood samples of the patient and evaluated whether exoantigen of *A. alternata* could be used for accurate immuno identification of systemic phaeohyphomycosis. They often play an important role as pathogens in osteomyelitis, pulmonary cutaneous and in keratomycosis (Farina et al. 2007) also reported a case of phaeoyphomycosis caused by *Alternaria alternata* in a kidney transplant patient. There are over 40 reported cases of human infection caused by *Alternaria* species (Shugar et al. 1981, Vivani et al. 1986, Mayser et al. 2002 and Chaidemenos et al., 1995). A majority of the reported cases occurred either in patients with severe underlying disease or in those receiving immunosuppressive drugs.

CASE REPORTS

Case 1:- A 35 year old male admitted with complaint of weakness, breathlessness, chest pain on right side since past 4 months. He had low grade fever during evening and hypoprotinaemia. His ESR was raised and sputum was positive for A.F.B. X-ray chest showed cavities on both apexes of lungs. He also complained of bleeding from mouth. He was diagnosed as a case of tuberculosis.
Case 2:- A 21 year-old male admitted in the hospital with the complaint of pain, fever and swelling above both the knee joint since last 2 months. He was a known case of rheumatic heart disease and was operated for the same during childhood. Second operation was done for valver defect, 15 days before the date of sample collection.

MATERIAL AND METHODS

Direct microscopy

2 ml. Of peripheral blood from the patients was collected by means of vein puncture using sterilized needle and syringe, transfered into sterilized 3 ml. of Sabouraud's dextrose broth bottle coated with heparin and incubated for 24 hrs at 28 ± 1^0 C. Blood smear was prepared and stained with lactophenol cotton blue and loopful of blood sample was streaked on Sabouraud's dextrose agar (SDA) with chloramphenicol and incubated at 28 ± 1^0 C for

7 days the fungus recovered was identified on the basis of direct microscopy, thermotolerance, macro and micromorphological characteristics and slide culture method.

In – vitro senstivity

In vitro sensitivity studies of ***A. alternata*** was conducted in antifungal assay medium (Himedia, Bombay) against antimycotics, (Ketoconazole, Fluconazole, Itraconazole, Clotrimazole using 1:3 fold) dilution method. The minimum inhibition concentration (MIC) of drugs at which no growth occurred after 24,48,72,96 and 168 hours at 28±1°C as per visual observation was recorded.

Serology studies

A. Preparation of exonatigens

Standard and Kaufman (1978) method used for preparation of exoantigen, *Alternaria alternata* isolated from peripheral blood of patients and maintained on SDA medium. 100 ml of Sabouraud's dextrose broth was dispended into flasks. Suspension of the micro-organism was prepared and filtered, and then the inoculum size was calculated by haemocytometer. 1ml of the inoculums was added aseptically in 250 ml of SDA broth and flasks were incubated at 28±1°C for 2 weeks. The method used for obtaining exo-antigen was Standard and Kaufman (1978). At the end of incubation, culture was killed by adding 1:5000 thimersol and then kept at 28±1°C for 48 hrs with shaking at regular intervals. Culture filtered thorugh whatman filter paper no.1. The filtrate antigen was concentrated 20 folds using vacuum pump.

B. Immunization

The primary immunization was through intramuscular route. 1 ml of antigen and 1 ml Freund's incomplete adjuvant was injected within the thigh muscle of rabbit in 1st, 2nd and 3rd week. The animal was bled for antiserum on 7th, 14th and 21st day 3 ml of emulsified antigen was given intravenously on 4th week. The 4th route was taken for secondary immunization in the ear vein of rabbit. The animal was bled for antiserum after 1 week and tested by ODD. The cross reactivity of the test antigen was done against homologous and heterologous antiserum from rabbit by ODD method.

C. Antigen and Antiserum analysis

The analysis of antigen and antiserum collected was performed by SDS-Polyacrylamide gel electrophoresis. The band was then recorded and analyzed. The protein and carbohydrate content of exoantigens were analysed by Standard Biurette and Anthrone's method respectively.

RESULTS

1. The direct microscopic examination of the blood sample revealed dark coloured, septate mycelium and dematiaceous spores. The morphological observation showed dark grey-brown to black colour on SDA medium. The conidia was multicelled with both transverse and longitudinal septa and had a dark brown colour. They were elongated and drumstick shaped. The conidia was brown to golden in colour (Figure 1 and Figure 2).

2. In vitro activity of four antimycotic drugs viz. Ketoconazole, Fluconazole, Itraconazole and Clotrimazole was tested against *Alternaria alternata.* Ketoconazole was found most effective drug showing no growth after 24 hrs of incubation. It gave MIC value of 1µg/ml, 3µg/ml, 10µg/ml, 30µg/ml after 48, 72, 96 hours and 7 days of incubation respectively. Itraconazole was able to inhibit the growth after 24 hours and gave MIC value of 30µg/ml, after 48 and 72 hrs and 100µg/ml after 96 hrs with no inhibition after 7 days of incubation. Fluconzaole inhibited the growth completely till 48 hrs of incubation of all the test concentration of the drug. Clotrimazole was least effective, it offered no inhibition of growth after 48,72,96 hours and 7 days of incubation even at 100µg/ml but the growth was completely inhibited at all concentration upto 24 hrs of incubation (Figure 3).

3. SDS–PAGE results of case no 1 showed two bands with molecular weight of 15 K Daltons and 67 K daltons. The 15 K dalton bands was however more intense (Figure 4). The ODD test showed development of specific immune response in experimental rabbits with *A. alternata* precipitation band could be detected after one week of challenge. Similar bands appeared upto vth week. *A. alternata* exoantigen showed humoral response after 1 week of immunization (Figure 5).

4 Cross reactivity of the four exoantigens, was tested against 2 strains of *Curvularia verreculosa, Cladosporium cladosporioaides and Alternaria alternata.* Specific antisera raised in rabbits. No cross reactivity was seen with antisera raised in rabbit aginst 2 strains of *Curvularia verreculosa* (CV I and CV II) and *Cladosporium* (Figure 6). Many additional bands could be detected particularly higher than 67 KD molecular weights as compared to the normal serum obtained from the same rabbit before challenge with the antigen which

exhibited just four bands. Significant increase in the level of IgG to all antigens as compared with the normal antisera.

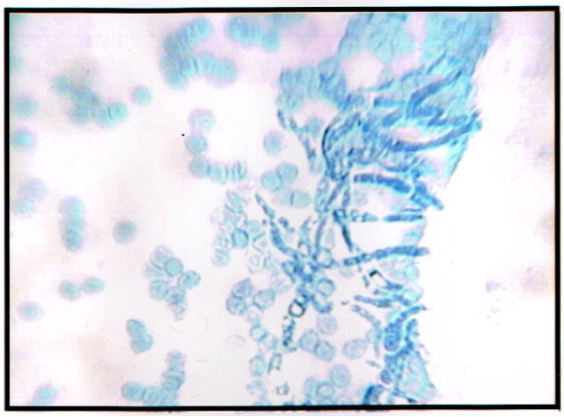

Fig.1. D.M. of blood of case no 1 cotton blue stained, 400 x showing dark coloured septate mycelium.

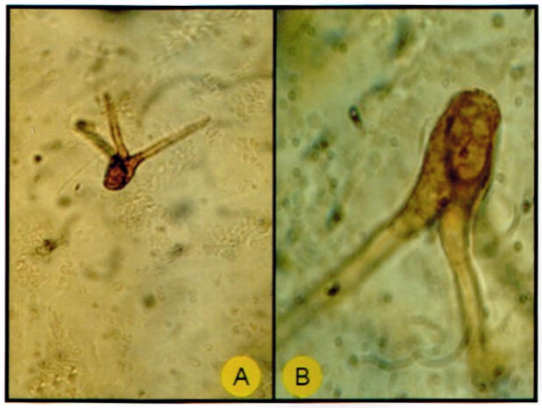

Fig. 2. D.M. of blood sample of case no 2(A) 200 x (B) 1000 x showing dark dematiaceous septate mycelium and cells.

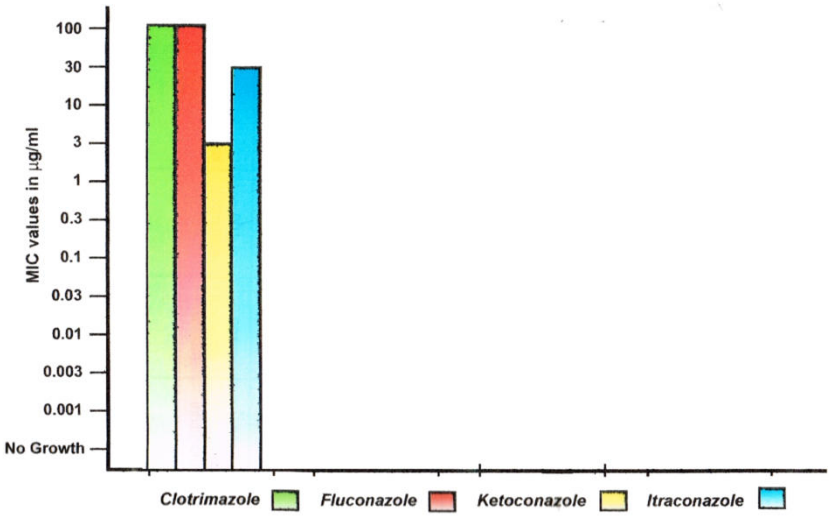

Fig. 3. Histogram showing the minimum inhibitory concentration values (MICs) of 4 antimycotics against A. alternata after 72 hrs of incubation on Antifungal Assay Agar (AAA) medium.

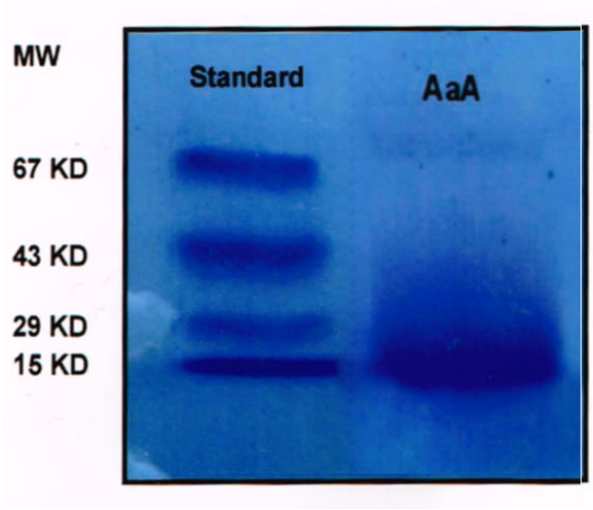

Fig.4. SDS-PAGE analysis of the Alternaria alternata exoantigen showed two bands with molecular weight of 15K Daltons and 67K daltons.

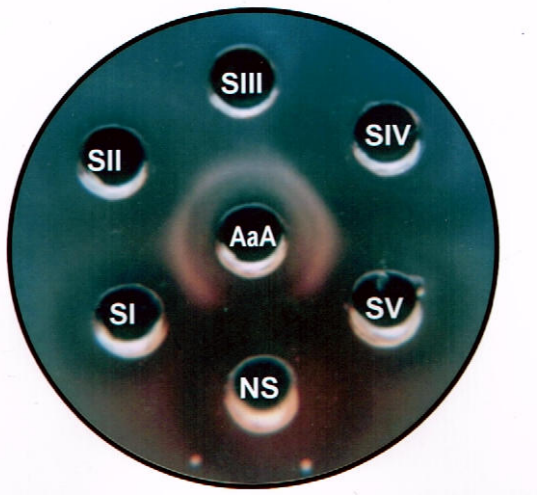

Fig. 5. Immunodiffusion of A. Alternata with normal serum and antiserum raised in rabbit 1-5 weeks.

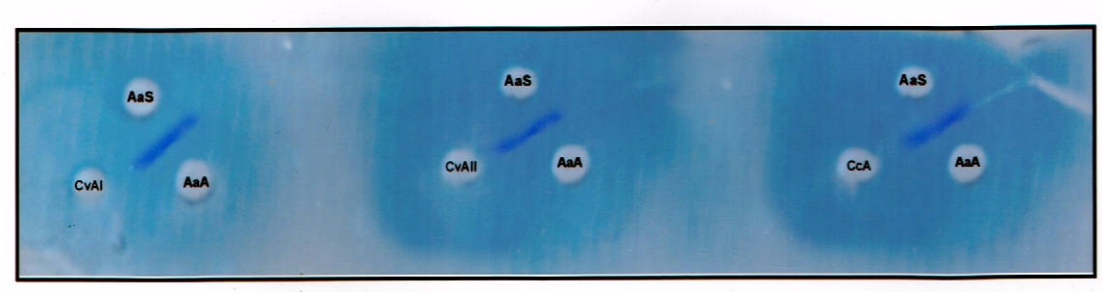

Fig. 6 : Cross reactivity of the four antigens, with Alternaria alternata antiserum of rabbit. No cross reactivity was seen seen with antisera raised in rabbit against 2 strains of Curvularia verreculosa (CV I and CV II) and Cladosporium cladosporioaides.

DISCUSSION

Alternaria alternata is the most frequently encountered species of *Alternaria* causing opportunistic mycosis. In the present investigation two patients of *A. alternata* had tuberculosis and one was with rheumatoid heart disease in which mortality was observed. Neumeister et al. (1994) reported *A. alternata* from cutaneous nodules. Mayser et al. (2002) reported a case of cutaneous alternariosis due to *A. alternata* in a renal transplant recipient where surgical treatment accompanied by prophylactic application of itraconazole resulted in complete cure. Similar case and treatment was also reported by Romano et al. (1997) in immunocompromised patients.

In the present study ketoconazole with the MIC of 10µg ml^{-1} was found to be the best effective drug against both the case tasted in vitro susceptibility test against *A, alternat.* A pathogen causing of onchomycosis showed that oxiconazole (0.1 µg/ml,) was very effective and ketoconazole (3µg/ml) also quite effective against the pathogen Naidu et al. (2000).

Schumacher et al. (1975) chemically characterized the culture filtrate antigen of *Alternaria tenis* by radiolabelling with 123-1. Staining patterns following electrophoresis in polyacrylamide revealed that antigen components had high polysaccharide content. Wijnands et al. (2000) reported that *A. alternata* produced one antigen that can be found under various growth conditions in extract of the water soluble portion of the mycelium and culture filtrate have been indentified the function of marker for those exposed to allergens of *A. alternata*.

Agar gel double diffusion test is a molecular tool helpful in the analysis of culture filtrate of the pathogen of serum of patients. Soluble exoantigen are revealed against specific antisera. In the present investigation similar procedure was applied with good result in presumptive identification of *A.alternata*. In

antigen of *A. alternata* two precipitin bands were observed with the rabbit antisera, with the humoral response being evident just after one weeks of challenge. Likewise Mukherjee (1989) detected that the exoantigen of *Sporotrichum pruinosum* could elicit the formation of antibody in the patient serum as well as in rabbit serum and gave specific lines of identity in the gel diffusion test. The normal serum of uninfected rabbit in the present study exhibited four bands, out of which three were intense prominent bands and one was weak band. Up to 9 bands were observed in the antiserum of rabbit challenged with exoantigen of *A. alternata* with molecular weight from 15 KDa to higher than 67 KDa with the gamma globulin band becoming more intense in immunized rabbit. Zrimsek et al. (2003) likewise reported the appearance of large amoung of degraded proteins on immunoblots of positive samples of rabbit antiserum of *Trichophyton mentagrophytes*. None of the bands observed in the antiserum of rabbit could be differentiated as specific marker for *A. alternata* because they were quite similar but whether any of the bands could be treated as specific marker for dematiaceous group needs further investigations. Sparkes et al. (1994) who investigated with humoral immune response infected with *M. canis* were also not able to find a maker 100% specific for infection. Similarly Zrimsek et al. (2003) none of the bands seen on specific marker for dermatophytosis. Exoantigen study exhibited specificity and could be used in the serodiagnosis of such patients. However it needs further trails in terms of cross reactivity and its reactivity with patients' sera.

ACKNOWLEDGEMENTS
It is my privilege to express the deep sense of gratitude to my guide late Dr. Jaishree Naidu. Authors are grateful to the patients also for their cooperation and bearing pain for us.

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