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Systemic Phaeohyphomycosis Caused by Cladosporium Cladosporioides: In Vitro Sensitivity and its Serological Diagnosis

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ABSTRACT
Phaeohyphomycosis is a clinical entity caused by dematiaceous fungi. *Cladosporium cladosporioides* is a phaeoid fungi commonly found in man's environment and has been reported to cause infection in man. We report a cause of pheohyphomycosis in a 60 year old male suffering from tuberculosis and gangrene. *C. Cladoporioides* was isolated from peripheral blood of the patient. The direct microscopic examination of the blood sample revealed the presence of dark color dematiaceous septate & branched mycelium. In-vitro antifungal sensitivity of *C. cladosporioides* against Ketoconazole, Fluconazole, Itaconazole and Clotrimazole performed by Polak's 1:3 dilution method. Ketoconazole was the most effective drug against the isolated strain with MIC 10 µg ml^{-1} after 96 hrs. of incubation. The exoantigen of *C. cladosporioides* was prepared and subjected to SDS PAGE (Polyacrylamide gel electrophoresis) analysis which revealed two bands of 15 and 67 K Dalton. Antisera was raised against the antigen and subjected to ODD (Ouchterlony's double diffusion) test which showed highly specific band of identity. No cross reactivity was observed against the exoantigen of *C. cladosporioides* with antisera of 2 strains of *Curvularia verruculosa* and one strain of Alternaria alternata. In this study it is concluded that cases of phaeohyphomycoses caused by *C. cladosporioides* can be rapidly diagnosed using exoantigenic method.
Keywords; opportunistic, phaeohypomycosis, *Cladosporium cladosporioides* , minimal inhibitory concentration (MIC)

INTRODUCTION

Phaeohyphomycosis is a general term for an infection by a number of pigmented fungi. Most of the organisms associated with phaeohyphomycosis are ubiquitous and have been received from vegetative matter and soil all over the world. Traumatic implantation of the etiologic agent and wound contamination by the organism are thought to be the primary modes of infection. Dimatiaceous fungi are an increasing cause of human disease (Rossmann et al. 1996). In the present paper we report a case of *Cladosporium cladosporiades* infection where it has been isolated from peripheral blood sample of the patient and evaluated whether exoantigen of *C. dadosporioides* could be used for accurate immuno identification of phaeophyphomycosis. Cutaneous phaeohyphomycosis due to *C. cladosporioides* has also been described [by Annessi et al., 1992, Vieira et al., 2001].

Case report

A 60 years old male was admitted in the hospital with the complaint of cough with chest pain since last two months. Patient also complained of weakness, loss of appetite and bodyache. Patient noticed fever during evenings since two months. He had a greenish discolouration and loss of sensation in left foot and slight oedema was present all were the body. In respiratory system, there was crepitation on both the lungs. He was diagnosed as a case of tuberculosis and gangrene in left leg.

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 ***C cladosporoides*** 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, ***Cldosporium cladosporoides*** 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

The direct microscopic examination of the blood sample showed dark coloured, dematiaceous, septate and branched mycelium. The fungus showed myceloid and regular colony on SDA medium. The centre of colony was white having a woody texture. While the peripheral zone was of olivaceous to iron grey colour. Conidia were smooth, pale olivaceous brown in colour. They were one celled, slightly tapering at one or both the ends. The fungus showed moderate growth at 28°C, slow at 37°C and no growth at 40°C. (Fig 1)

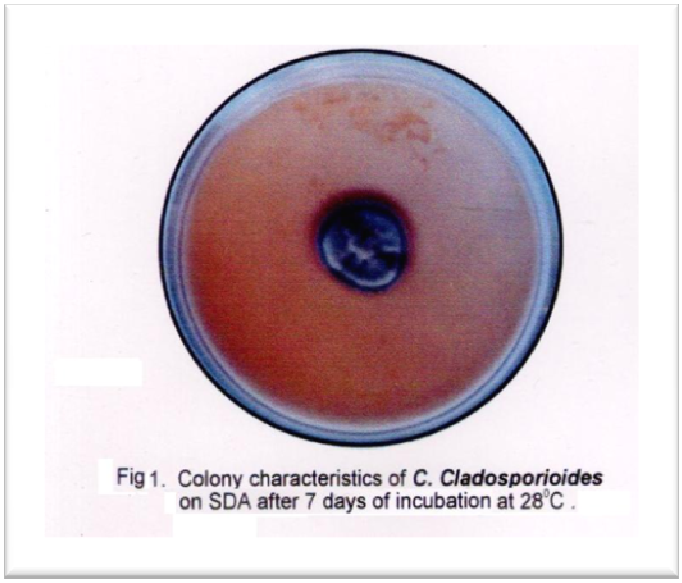

Fig 1. Colony characteristics of *C. Cladosporioides* on SDA after 7 days of incubation at 28°C .

In vitro activity of four antimycotic drugs viz Ketoconazole, Fluconazole, Itraconazole and Clotrimazole was tested against Cladosporium cladosporioides. Out of the four drugs tested, only Ketoconazole showed Fungistatic activity inhibiting the growth completely upto 48h of incubation. It gave MIC value of 30µg/ml

upto 96h and of 100µg/ml of the 7 days of incubation. The other three drugs i.e. Clotrimazole, Fluconzaole and Itraconazole failed to inhibit C.cladosporioides at all the tested concentration after 48, 72, 96 hours and 7 days of incubation. (Fig. 2)

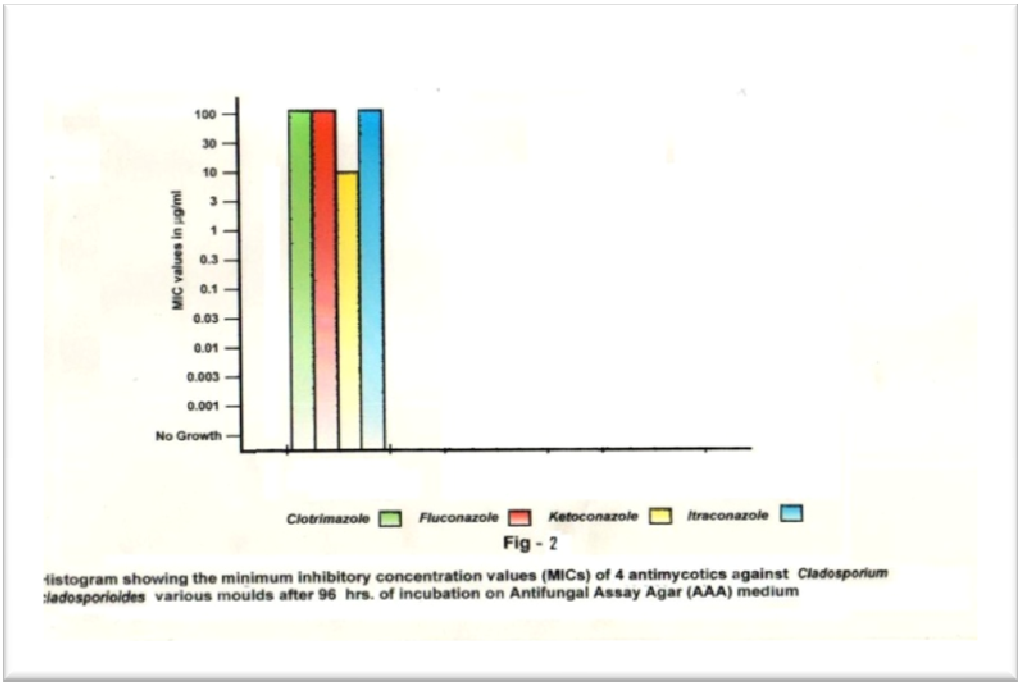

Fig - 2

Histogram showing the minimum inhibitory concentration values (MICs) of 4 antimycotics against *Cladosporium cladosporioides* various moulds after 96 hrs. of incubation on Antifungal Assay Agar (AAA) medium

SDS PAGE result of the exoantigen of Cladosporium cladosporioides showed two bands with molecular weight of 15K daltons and 67K daltons. However the 15Kd band as mere intense (Fig. 3).

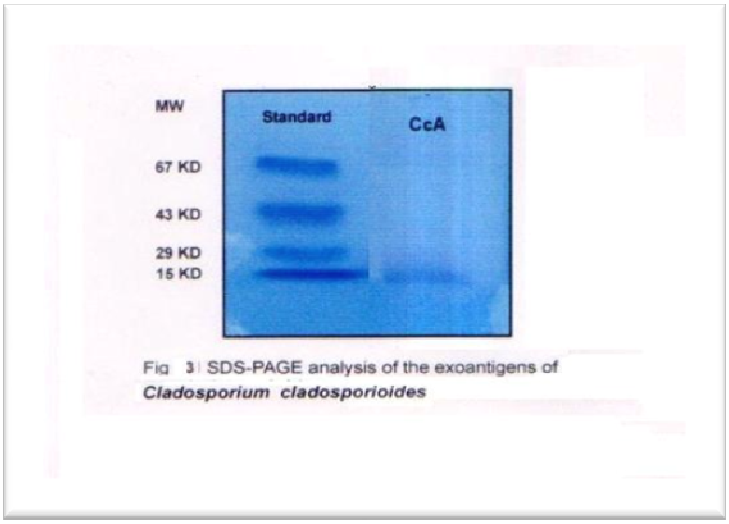

Fig 3 SDS-PAGE analysis of the exoantigens of *Cladosporium cladosporioides*

The ODD test showed development of specific immune response in rabbits challenged with exoantigen of C. cladosporioides. Precipitation band could be detected after one week of challenge. Similar bands appeared upto Vth week. Cross reactivity of C.cladosporioides was tested against specific antisera raised in rabbits. C.cladosporioides antigen showed specific reactivity against their own antiserum but not with other test antiserum raised in rabbit (Fig.4). The antisera developed in rabbits against C. cladosporiodes showed many additional bands higher than 67 Kd molecular weight as compared to normal serum obtained from the same rabbit before challenge with the antigen, which exhibited just four bands. There was significant increase in level of Immunoglobulin G to the exoantigen as compared with the normal antisera. (Fig.5).

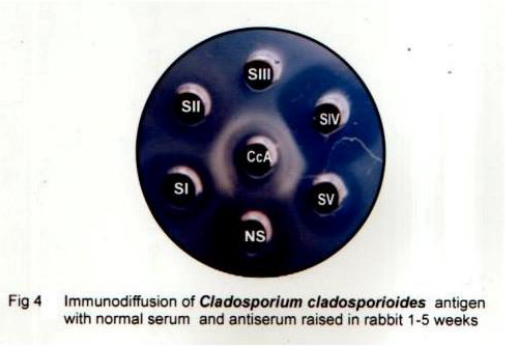

Fig 4 Immunodiffusion of ***Cladosporium cladosporioides*** antigen with normal serum and antiserum raised in rabbit 1-5 weeks

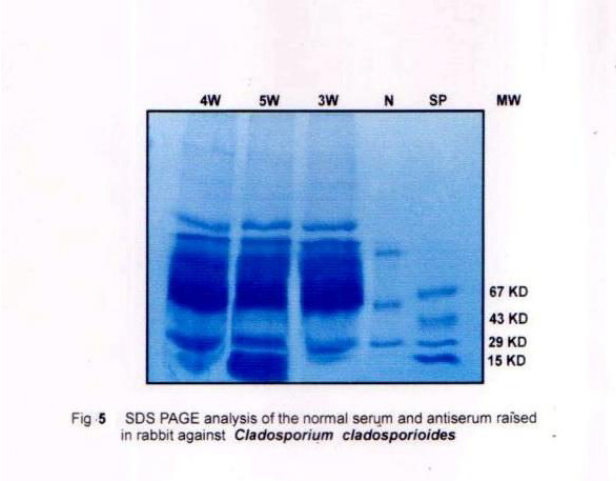

Fig 5 SDS PAGE analysis of the normal serum and antiserum raised in rabbit against ***Cladosporium cladosporioides***

DISCUSSION

Cladosporium cladosporioides is a rare etiological agent of human infection. It was previously isolated in a human immune deficiency, virus (HIV) seropositive patient as an opportunistic pathogen at the site of skin testing (Drabick et al, 1990), in a patient affected by Pemphigus vulgaris causing also cutaneous lesions (Annessi et al.1992). In the present investigation, a fatal case of Cladosporium cladosporiodes was recorded in a patient with tuberculosis and gangrene. Dixon et al. (1989) also examined the presence of potentially pathogenic fungi, 55% of the corneal donar was positive for fungi Cladosporium species. Nand Lal Sharma et al. (2002) reported 23 patient with sub cutaneous phaeophyphomycosis.

In the present study ketoconazole was found to be the most effective drug with the MIC value of 30µg ml-1 after 48 hrs. of incubation. Ketoconazole moderately affects C. cladosporiodes, whereas other azole was ineffective against the pathogen. McGinnis(1986) and Pasarell (1990) reported that Itraconazole was active against dark fungi.

In the present investigation culture filtrate antigen of C. cladosporioides was examined for being used as tools for serodiagnosis of this infection. The C. cladosporiodes exoantigen was found to be glycoproteinaceous in nature and gave weak protein bands of 15 kd and 67 kd compared to other dematiaceous fungi. Agar gel double diffusion test is a molecular tool helpful in the analysis of culture filtrate of the pathogen or serum of patients (Howard, 1983) similar procedure was applied with good result in the presumptive identification of C. cladosporioides. Two precipitin bands were obtained with the rabbit antisera, with the humoral response being evident just after one week of challenge with antigen. Likewise, Mukherjee (1989) detected that the exoantigen of Sporotrichum pruinosum could elicit the formation of antibody in the patients serum as well as in rabbit serum and gave specific lines of identity in the gel diffusion test. The normal serum of uninfected rabbit exhibited four bands, in which three were intense prominent bands and one weak band. Upto 9 bands were observed in the anti serum of rabbits with molecular weights from 15 kDa to higher than 67 kDa with the γ immunoglobulin bands becoming more intense in immunized rabbit. Zrimsek et al. (2003) likewise reported the same results from the fungal mat of Trichophyton mentagrophytes. Serology is a useful tool in the diagnosis of deep mycoses. Humoral response was evident after one week of antigen immunization. It is used in the serodiagnosis of patients.

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