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Research Article

AN OPPORTUNISTIC FUNGAL CONSORTIUM CAUSES SUPERFICIAL SKIN MYCOSIS: A CASE STUDY

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ABSTRACT

Opportunistic fungal pathogens live in soil as saprobes. They become pathogenic when the immune system of the host is compromised or suppressed. Present investigation is a case study carried out on a 65 years old patient suffering from acute skin infection in the lower part of the leg. Fungal samples were isolated from the patient suffering from acute skin infection in the lower part of leg. All the fungal isolates isolated during the study had clinical significance.

Keywords: *Opportunistic Fungal Pathogens; Skin Mycosis*

INTRODUCTION

Opportunistic fungal pathogens are generally soil inhabiting species where they live as saprobes but they become pathogenic when the immune system of the host is compromised or suppressed. With the emergence of fungi that are resistant to many of the anti fungal drugs available, identification of the significant clinical isolates is important as delays in the initiation of appropriate therapy often correlates with poor outcome. It has therefore, become essential to have rapid and accurate methods for identification of fungi that can easily be implemented in a routine diagnostic microbiology laboratory for guiding early appropriate therapy and prevent mortality. Conventional methods for identification of these pathogens are relatively insensitive showing false negative results. Fungal pathogens have been reported to cause infections in human beings suffering from various diseases such as cancer, diabetes and AIDS. These fungal infections are mostly caused by saprophytes that are generally harmless in the environment but become opportunistic when the immune system of patients become compromised or suppressed. The main cause of the fungal infection is occupational hygiene. The infection is more prevalent among farmers than other professions (Perfect, 2001; Spiewak *et al.*, 2000). Farm work is generally associated with developing higher risk of superficial skin mycosis, because they mostly deal with cattle, pets, and garden soil etc. The patients suffering from the fungal infections show symptoms like unpleasant skin changes, secondary allergization, inflamed skin, formation of skin pustules, etc which also promotes other microbes to invade the body through this infection (Wanke *et al.*, 2000; Bakheshwain *et al.*, 2011). In the present investigation a case study was carried out on a 65 years old patient suffering from acute skin infection in the lower part of the leg, case profile of the patient was recorded with various parameters such as age, occupation, medical case history etc.

MATERIALS AND METHODS

The study was carried out from the old patient with a suspicion of fungal infection on lower part of the leg. A total of five fungal isolates were isolated from the infectious part.

a) Physical Examination

The symptoms showed scattered lesions with skin cracking, peeling and with inflammation at the margins. Itching and irritation was recorded in severe infection areas. Small papules, scaly lesions and swelling of deeper tissue of skin were observed.

b) Collection of the Samples

Fungal samples were collected by scraping of the skin and collected into folded dark paper squares. Secured the dark paper squares with a paper clip in a sterilized way and subjected for KOH test. Selected samples were inoculated on Sabouraud Dextrose Agar media supplemented with Chloramphenicol and incubated at 28 ±2ºC.

c) Morphological and Microscopic Identification

The fungal cultures were identified on the basis of morphological characteristics like shape of the colony, size, elevation, border margins, surface of the colony and pigmentation. Microscopic identification was done by observing the presence or absence of macro or micro conidia, arrangement of spores and shape of conidia (St-Germain and Summerbell 2003; Hoog *et al.*, 2000).

d) Molecular Identification

The molecular characterization of the isolates was done based on amplification of the ITS regions followed by sequencing. Fungal mycelial disc was inoculated into 125 ml of Sabouraud's dextrose broth at 30°C for 4-5 days at 125 rpm, filtered through muslin cloth and dried to harvest the cells for DNA extraction. DNA extraction was performed according to (Saghai, 1984). 500mg of dried mycelial mass was ground in pre cooled mortar using liquid nitrogen. Powdered mass was put in CTAB extraction buffer (100mM Tris HCl pH 8.0, 20mM EDTA, 1.4mM NaCl, 2%CTAB) and incubated at 60°C for 1 hour. After incubation equal volume of chloroform: isoamylalcohol in 24:1 ratio was added followed by centrifugation at 5000 rpm for 10min. Supernatant was extracted with phenol: chloroform: isoamylalcohol 25:24:1 and centrifuged at 10,000 g for 10 minutes. Aqueous phase was collected, mixed with an equal volume of chloroform and centrifuged at 10000g for 10min. Aqueous phase was precipitated with chilled ethanol and precipitates were dissolved in TE buffer and stored at -20°C. DNA was purified and quantified. For RNase treatment DNA samples were incubated at 37°C for three hours. The DNA was quantified and purified; genomic DNA (1µl/25ng conc.), $MgCl_2$ (2µl), $Mgcl_2$ buffer (2.5µl), dNTP's(2.5µl), MilliQ(15µl), ITS1 and ITS4 (1µl+1µl), Taq polymerase (1U). PCR was performed for 30 cycles; 95ºC for 5 min, 95 ºC for 30 sec, 55 ºC for 60 sec, 72 ºC 1 min, 72 ºC for 7 min. The ITS1 and ITS4 Primers used were: ITS1: 5'-TCC GTA GGT GAA CCT TGC GG-3'and ITS4: 5'TCC TCC GCT TAT TGA TAT GC-3'

Agarose Gel Electrophoresis and Sequence Analysis

Amplified DNA was examined in 2% Agarose gel, stained with ethidium bromide (10mg ml^{-1}) and then seen under UV transilluminator. Desired amplicons were further purified by using AXYGEN purification kit and processed for sequence analysis.

RESULTS AND DISCUSSION

Results

The present study focused on the fungal consortium causing infection in the patient under study. Case profile of the patient was recorded with respect to various parameters such as age, occupation and medical case history. Fungal samples were isolated from 65 years old patient suffering from acute skin infection in the lower part of leg. By occupation he was a farmer (Table1). The patient showed symptoms like unpleasant skin changes, secondary allergization, inflammation of skin and formation of skin pustules (Figure 1). Fungal samples were brought to the laboratory for isolation of opportunistic fungi and were grown in Sabouraud Dextrose Agar medium at 28 ± 2 º C. After regular monitoring fungal cultures were preliminary identified based on shape of the colony, size, elevation, border margins, surface of the colony and pigmentation. Cultural characteristics showed Accession ANJ1 to be dark green cottony colony with white margins at centre; Accession ANJ2 was leathery green with white margins; Accession ANJ3 was leathery brown in colour; Accession ANJ4 was dark brown leathery colony and Accession ANJ5 was off-white cottony colony with light pink margins. The cultures were further subjected for microscopic study of the shape and size of the conidia. *Epicoccum sp*. comprises short conidiophores bearing multicellular spherical to club shaped macroconidia, transverse and longitudinal septa, dark yellow hyphae with brown

conidia. Conidia of *Pestalotiopsis* are long fusiform, elliptic-clavate and slightly curved. The microscopic study of the *Aspergillus flavus* included various characters including conidiophores, vesicles and conidia. Although the fungal isolates could be identified upto certain extent on the basis of macro and micro morphological characteristics but it becomes important to characterize them at molecular level. Conventional methods for identification of these pathogens are relatively insensitive showing false negative results. Numerous targets within fungal genome have been evaluated using sequence areas of rDNA gene complex for the rapid detection and identification of fungi. During the present study also, fungal isolates were further characterized at molecular level based on ITS1 and ITS4 regions. Fungal genomic DNA was amplified using ITS1 and ITS4 primers. The amplified product size ranged from 530-550 bp and was subjected to sequencing (Figure 2). The obtained DNA sequences were BLAST and compared by Clustal W. Fungal samples were identified to species level through DNA sequencing of the nuclear internal transcribed spacer (ITS) region for use in BLAST-based similarity as follows; (ANJ1) *Aspergillus flavus* (99%), (ANJ2) *Emericella quadrilineata* (99%), (ANJ3) *Epicoccum sp.* (99%), (ANJ4) *Epicoccum nigrum* (100%), (ANJ5) *Pestalotiopsis sp.* (100%). The nucleotide sequences obtained showed maximum similarity with following species by evaluating bootstrap values (Figure 3). The phylogenetic analyses were carried out to identify the clinically important fungi by MEGA software (Nilsson *et al.*, 2009).

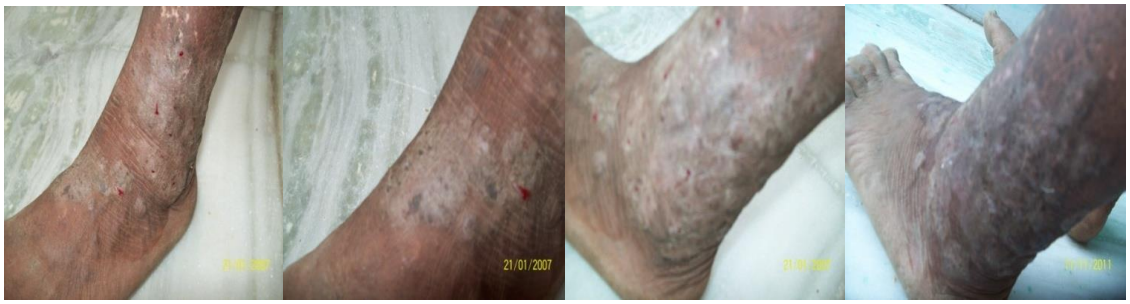

Figure 1: Symptoms shows acute fungal infection at the lower part of the leg of the patient

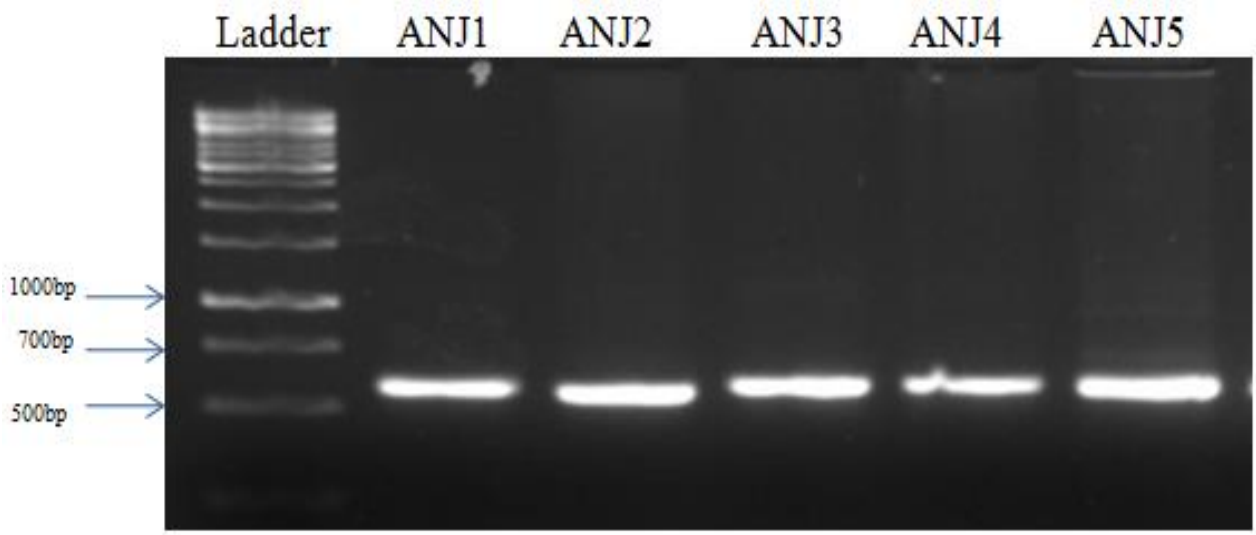

Figure 2: Amplification of ITS region of genomic DNA of clinically important fungal isolates

Table 1: Case history of patient suffering with infection by fungal consortium

S.No	Patient's name	Date of sample collection	Residence	Gender	age	Infected body part	Total Cultures isolated	Fungal appearance in plate	Identified fungal cultures
1.	Puran chand	5.04.2011	Pallan, (Billawar-J&K) India.	Male	65	Skin of lower leg	ANJ1	Dark green cottony colony with white margins	*Aspergillus flavus*
							ANJ2	Leathery green with white margins	*Emericella quadrilineata*
							ANJ3	Leathery brown colony	*Epicoccum sp.*
							ANJ4	Dark brown, leathery colony	*Epicoccum nigrum*
							ANJ5	Off-white cottony colony	*Pestalotiopsis sp.*

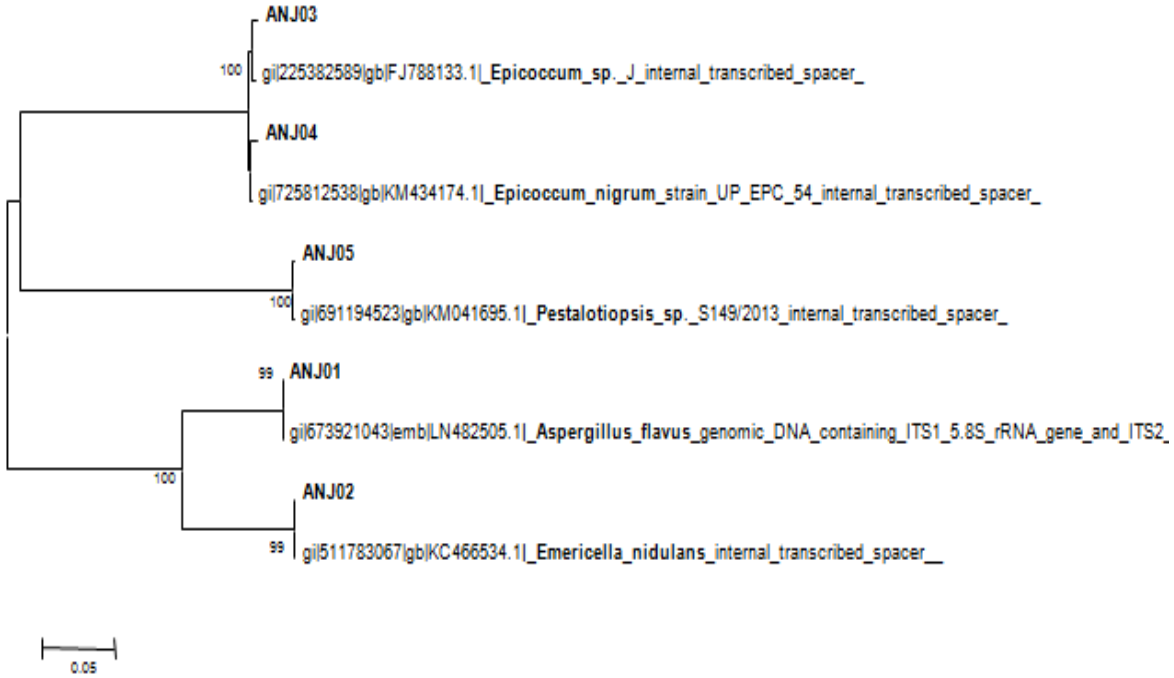

Figure 3: Phylogenetic Tree of clinical isolated fungi showing inter-relationship based on ITS sequences

Discussion

All the five fungal isolates have been reported as opportunistic fungi. The cultures were identified as *Aspergillus flavus, Emericella nidulans, Epicoccum sp, Epicoccum nigrum,* and *Pestalotiopsis sp.* The *Epicoccum sp* belongs to Phaeohyphomycetes and is considered under Dematiaceous fungi known to infect cornea and is opportunistic. Generally it is found in soil, air, and water and rotting vegetation and become opportunistic pathogen by causing allergy to the skin of the immunocompromised patients (Iracema *et al.*, 2001). Portony *et al.*, (1987) and Horner *et al.*, (1995) reported *Epicoccum sp.* from air sampling. Latest studies on *Epicoccum* sp. indicates it as skin allergen. On the basis of comparable degree of skin reactivity, it was observed that *Epicoccum sp.* shows 70% skin sensitivity as compared to other fungal extracts. Dixit *et al.*, (1992), have identified allergens on the basis of immunoblotting from both

spores and mycelium. The prevalence of the fungal spores was reported in urban and rural environment and widely distributed as saprophyte but becomes allergenic when in contact with skin, through inhalation, clothes, food stuff etc. The functional behaviour of *Epicoccum nigrum* exhibits synthesis of an extracellular fungal polysaccharide called epiglucan. *Epicoccum nigrum* has been reported to colonize nasal sinus and cause allergic fungal sinusitis (AFS) (Schmid *et al*., 2001). Therefore, toxigenic molds are the risk factors for the adverse human health problems including neuropsychological disorders. *Pestalotiopsis* has received consideration as saprophytic fungi of plants and is rarely found in animals but becomes opportunistic in immunocompromised patients (Pounder *et al*., 2007). The species of *Pestalotiopsis* are widely distributed and isolated from soil. They are also known as potential human and animal pathogen, which cause several chronic diseases in human beings (Maharachchikumbura *et al*., 2011). *Pestalotiopsis* species have been isolated from the human sinuses, fingernails, bronchial biopsy, eyes, scalp, bone marrow transplant recipients, cancer patients, diabetic and immunocompromised patients (Sutton, 1999).

Aspergillus sp. are mostly found in the air, house dust, food, soil hay, damaged wooden doors, etc more often cause allergy to a person having low immune system the disease caused by this known as Aspergillosis (Verweij *et al*., 2008). Majority of the fungi are medically important and *Aspergillus sp* are more prevalent as invasive etiological agents of mycosis (McClenny, 2005). In India, a case report of 60 year old male patient of Uttar Pradesh exhibited a chronic obstructive pulmonary disease and caught to dystrophy of all five nails of the right hand, the patient history supported that he was a farmer by occupation and his daily routine associated with soil based job the prevalence of onchomycosis is due to *Emericella nidulans*. They are considered as non dermatophytic fungi but are being reported as etiological agents of onchomycosis (Gugnani *et al*., 2008). It was also reported that *Aspergillus flavus* cause *Aspergillus* myositis due to liver transplantation. *Aspergillus flavus* is also an important agent to cause keratitis. The main reason to become pathogenic is due to the production of some potent mycotoxins, known as aflatoxin that is predominately harmful to humans and animals. Various environmental conditions influence such as temperature, pH and nutrient sources. The isolates preliminarily identified as *Aspergillus flavus* on the basis of macroscopic and microscopic morphology. The final conformation for identification is based on molecular level studies by using ITS1, ITS2, ITS3 and ITS4 primers. The amplicons obtained were further analyzed by performing nucleotide sequencing and DNA alignment was further carried out by ClustalW. The DNA based identification gives more significant result by using ITS1 and ITS4 primers which gives more authentic result and does not depend on morphological based identification. In fungi which are non sporulating and difficult to identify by conventional methods, molecular based study could over rule this drawback (Rakeman *et al*., 2005). The ITS based primers give the full length of internal transcribed spacer region of the species, which is further used to study the phylogenetic relationship with others fungi (Iwen *et al*., 2002). The comparative studies have been reported to be conducted on the basis of BLAST and Phylogenetic analysis was performed to differentiate the fungal strains at species level using MEGA software (Leema *et al*., 2010). *Aspergillus* and some others fungal isolates have also been reported from immunocompromised patients and explored for various parameters to identify and get comparative nucleotide based sequences by using ITS1 and ITS2 primers (Balajee *et al*., 2009). Bagyalakshmi *et al*., (2007), reported that nucleotide polymorphism of internal transcribed spacer (ITS) region of ocular isolates of *Aspergillus flavus* from the same patient showed similar nucleotide polymorphism. Thus, DNA sequencing proves that molecular based technique gives more reliable results to screen the genetic similarity among other fungal isolates.

Conclusion

The present study concludes that the fungal infections are mostly caused by saprophytes that are generally harmless in the environment but become opportunistic when the immune system of patients become compromised or suppressed and working condition greatly influence the development of fungal infection. All the fungal isolates isolated during the study have clinical significance. The case history of patient supported the relevance of the isolated fungi and to improve the diagnostic value the fungi were

characterized by various identification parameters such as microscopic, macroscopic, and molecular based studies that made the present work more reliable.

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