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SHORT COMMUNICATION

Rhizopus homothallicus Causing Invasive Infections: Series of Three Cases from a Single Centre in North India

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Abstract Mucormycoses are opportunistic fungal infections with a high mortality rate. *Rhizopus oryzae* is the most common agent implicated in human infections. Although *R. homothallicus* has been previously reported to be a cause of pulmonary mucormycosis, it is the first time that we are reporting as a causative agent of rhino-orbital and cutaneous mucormycosis.

Keywords Mucormycosis · Invasive · *Rhizopus homothallicus* · Rhino-orbital · Cutaneous · Pulmonary

Introduction

Mucormycoses are opportunistic fungal infections caused by fungi belonging to the order Mucorales [1]. These infections are life-threatening and are more common among patients with poorly controlled diabetes mellitus and patients with haematological malignancies. Increasing use of immunosuppressive drugs and prolonged antifungal therapy has led to an increase in the incidence of mucormycosis [2]. Rhizopus is the most common genus among Mucorales causing human infections with *Rhizopus oryzae* being the most frequent species implicated [3]. Here we report three cases of mucormycoses caused by an emerging species *Rhizopus homothallicus*.

Case Reports

Over a period of four years, we isolated *R. homothallicus* from three patients. The first patient was a 53-year-old female with features suggestive of rhino-orbital mucormycosis. The second patient was a 50-year-old female with complaint of an erythematous lesion with serous discharge on left side of face diagnosed as cutaneous mucormycosis (Fig. 1a). The third case was a 60-year-old male, known case of chronic myeloid leukaemia (CML) with pulmonary mucormycosis. Demographic information, clinical presentation, treatment and outcome have been described in detail in Table 1.

Biopsy samples received from all the three cases showed ribbon-like broad aseptate hyphae on histopathological examination. Direct examination in calcofluor stain with 10% KOH showed broad, aseptate, refractile hyphae with branching at right

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Table 1 Demographic and clinical details of all three cases

Case no.	Age/sex	Area of residence in India	Occupation	Time of presentation	Predisposing factor/s	Presenting complaints with duration	Radiological findings	Sample type	Antifungal treatment	Side effects due to antifungal agents	Outcome
1.	53/F	Delhi	Housewife	June 2013	Diabetic ketoacidosis	Nasal block, headache, facial pain and proptosis × 3 days	CT head showed oedematous and fluid filled ethmoid sinus with erosion of peri-orbital bone margins	Nasal tissue biopsy	Liposomal amphotericin B 50 mg/day × 4 days	Nil	Expired after 4 days of antifungal initiation
2.	50/F	Bihar	Farmer	April 2013	Diabetes	Erythematous lesion on face with mild serous discharge × 2 years	NA	Skin biopsy	Conventional amphotericin B 50 mg/day × 21 days	Nil	No relapse after 6 months of follow-up
3.	60/M	Uttar Pradesh	Farmer	July 2016	Diabetes, CML	Fever × 15 days chest pain, breathlessness × 3 days	CT chest revealed a well-circumscribed dense lesion in the left lung lower lobe	BAL, biopsy from mass in left bronchus	Liposomal amphotericin B 50 mg/day × 17 days	Nil	Expired after 17 days of antifungal initiation

angles (Fig. 1b). The samples were cultured on Sabouraud's dextrose agar (SDA) containing gentamicin and incubated at 25 and 37 °C. After 3–4 days of incubation, fast growing, cottony, white colonies were seen on the obverse with no pigmentation on the reverse side of the tubes. The colonies turned grey after prolonged incubation for two weeks. The colonies were teased and stained with lactophenol cotton blue. Microscopy showed poorly developed rhizoid tufts from which arose few lateral sporangiophores measuring 100–150 µm in length. These bore a few globose sporangia with scant number of angular to globose sporangiospores. A few globose, hyaline, intercalary chlamydospores were also present. There were a large number of golden brown zygospores measuring 60–100 µm in diameter with stellate spines on their walls. The suspensor cells were uneven with the zygospores attached to the larger, globose suspensor cell (Fig. 2). The isolates were thermotolerant and were able to grow when incubated up to 48 °C. Based on these characteristics, the three isolates were presumptively identified as *Rhizopus homothallicus* [4].

The identity of one of the isolates was further confirmed by polymerase chain reaction (PCR) and sequencing of the internal transcribed spacer (ITSs 1 and 2) region. DNA was extracted from the isolate using the Chelex method, and PCR was performed by method as described elsewhere [5, 6]. The purified

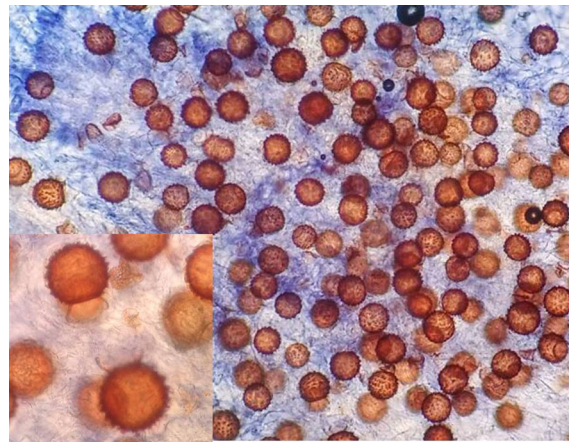

Fig. 2 *Golden* to *brown* coloured zygospores having remnants of single suspensor cell typical of *Rhizopus homothallicus* (low power). The *inset* picture shows the similar zygospores at high power

amplicons were further sequenced using the BigDye terminator cycle sequencing ready reaction kit, version 3.1 (Applied Biosystems, Foster City, CA) with primers ITS1 and ITS4. The reaction products were analysed on Genetic Analyzer 3130 (Applied Biosystems). The sequences thus obtained were compared with those in GenBank using the basic local alignment search tool (BLAST). Sequence showed 98% identity with ex-type strain of *R. homothallicus* (CBS 336.62) with GenBank accession number KR091552.

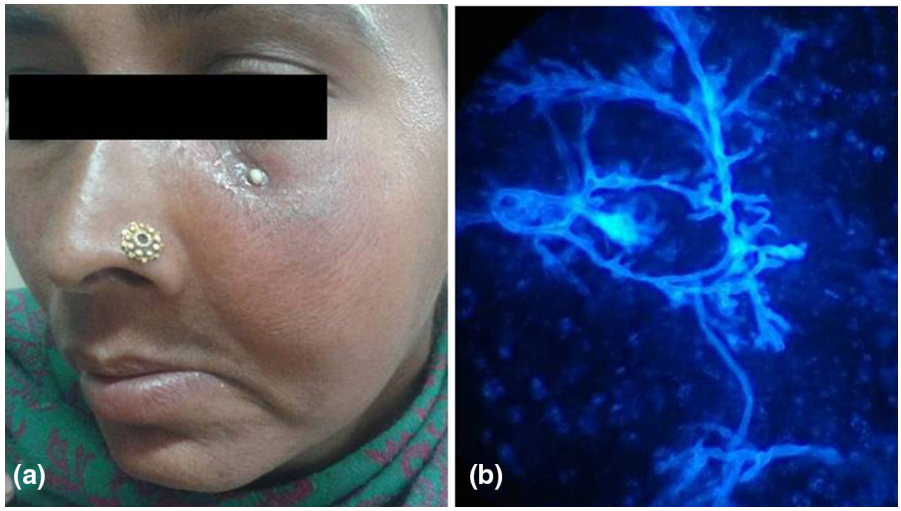

Fig. 1 **a** Erythematous lesion on *left side* of face with serous discharge and **b** calcofluor stain with 10% KOH showing broad aseptate hyphae (×40 magnification)

Discussion

The Mucorales belong to a distinct phylum called Glomeromycota [1]. Fungi belonging to this phylum are characterised by the formation of wide, ribbon-like, hyaline, aseptate hyphae and sexual reproduction with the formation of zygospores [3]. Human pathogens of the phylum are grouped in the class Mucorales. The class is further subdivided into two orders, the Mucorales and Entomophthorales [1]. Majority of human infections are caused by Mucorales. They have a worldwide distribution, produce abundant sporangiospores enclosed in a sporangium and produce zygospores from two opposed suspensors originating from different hyphae [1]. The pathogenic species are vasotropic and cause acute angio-invasive infections which are frequently fatal especially in the immunocompromised patients [3]. Though Mucormycosis is the term used to categorise infections caused by members of the order Mucorales, it is frequently used interchangeably with mucormycosis in the published literature [1].

The predisposing risk factors for developing mucormycosis include poorly controlled diabetes mellitus (with or without diabetic ketoacidosis), haematological malignancies (with or without stem cell transplantation), trauma, prolonged and severe neutropenia (absolute neutrophil count <1000 cells/µl for one week or more), iron chelation with deferoxamine, prolonged use of corticosteroids, intravenous drug use, immunodeficiency (HIV), protein calorie malnutrition and neonatal prematurity [2]. Prolonged use of antifungal therapy like caspofungin and voriconazole lacking activity against Mucorales is an emerging predisposing condition in recent years [3].

Rhino-cerebral mucormycosis, which represents 39% of infections by Mucorales, is common in patients with diabetes mellitus [7]. Pulmonary mucormycosis (24%) is more common in neutropenic patients and patients who have undergone stem cell transplantation and have graft versus host disease. Other sites of infection include skin (19%) and gastrointestinal tract [8]. Disseminated mucormycosis can occur from any of the primary sites though lung is commonly associated. Renal mucormycosis is quite rare, and few cases have been reported in renal transplant recipients and intravenous drug users [2]. In India, Chakrabarti et al. [9] have described an overall prevalence of rhino-orbito-cerebral mucormycosis (48%), followed by pulmonary mucormycosis (17%), gastrointestinal mucormycosis (13%), cutaneous mucormycosis (11%), renal and disseminated mucormycosis (5% each).

The most common species of the Mucorales recovered from clinical specimens belong to the genus *Rhizopus* followed by genera such as *Lichtheimia* and *Mucor*. These account for 70–80% of the cases of mucormycosis reported [10]. Based on phenotypic features (Schipper et al.) and genotypic characteristics (Abe et al.), the genus Rhizopus is classified into three groups, namely *R. oryzae*, *R. microsporus* group and *R. stolonifer* group [11–13]. *Rhizopus oryzae* and *R. microsporus* var. *rhizopodiformis* are the major human pathogens with the former being implicated in 90% of cases of rhino-orbito-cerebral mucormycosis [3]. Other important species include *R. schipperae, R. microsporus* var. *azygosporous* and *R. homothallicus* [3, 4].

Colonies of Rhizopus show rapid growth and have coarse and floccose aerial mycelia with pigmented rhizoids, brown unbranched sporangiophores and terminal globose sporangia bearing numerous sporangiospores [3, 11]. Zygospores are usually formed between oppositely oriented strains or heterothallic strains. They are large, range in size from 40 to 104 µm, bright brown to dark yellow brown in colour and have stellate, spiny projections on the walls [3]. Homothallic strains producing abundant zygospores include *R. homothallicus*, *R. sexualis* and *R. americanus* [13]. *R. sexualis* is a plant pathogen which causes rot of soft fruits especially strawberries [14]. The soil isolate *R. americanus* was earlier considered a variety of *R. sexualis,* but molecular studies by Ruyong et al. have proved it to be a distinct species [13].

R. homothallicus was first described by Hesseltine and Ellis in 1961 based on the ex-type strain NRRL 2538 (CBS 336.62). This strain was isolated from a soil sample collected in 1956 from a tropical desert at Zacapa station in Guetamala [4]. This strain no longer produces zygospores [12]. A second isolate, which is also considered a type strain (CBS111232, NRRL 2935), was collected from a soil sample from Lucknow, India, in 1961 (www.mycobank.org). The soil and environmental isolates produce abundant sporangiophores, sporangia and sporangiospores. These isolates lose their ability to sporulate when maintained at laboratory conditions [12]. *R.*

homothallicus has two varieties: *R. homothallicus* var. *homothallicus* and R. *homothallicus* var. *indicus*. The fungus is thermotolerant and can grow at 48 °C unlike other homothallic species [12]. Earlier considered an environmental isolate, *R. homothallicus* has recently been recognised as a human pathogen. Chakrabarti et al. were the first to describe invasive mucormycosis due to *R. homothallicus*. They isolated the fungus from two cases of pulmonary mucormycosis with cavitary lesions [15]. The present case series is the first to describe *R. homothallicus* as a cause of rhino-orbito-cerebral mucormycosis and cutaneous mucormycosis.

Structures similar to zygospores are also produced by *R. microsporus* var. *azygosporus* [3]. These are Azygospores and are round, pale to dark brown and have conical spines. Their size ranges from 30 to 70 µm. Unlike zygospores, these are produced on a single, well developed suspensor. The absence of two opposing suspensors indicates that Azygospores are formed as a result of parthenogenesis. Formation of Azygospores is best seen at 28 °C [16]. *R. microsporus* var. *azygosporus* is also known to cause human infections. Though rare, the organism has been reported to cause cutaneous and gastrointestinal mucormycosis [17, 18].

Though diagnosis of mucormycosis relies on histopathology and culture, identification of the isolate is time-consuming and largely depends on expertise of the mycologist. Molecular diagnostic tests are rapid and reliable and are widely accepted for accurate phylogenetic identification. Targets for molecular diagnosis include conserved genes like 28S rDNA, actin and EF-1alfa genes and the more variable internal transcribed spacer (ITS) region [19]. The ITS region is commonly used since it allows identification up to the species level even among closely related taxa [20]. Sequencing of the ITS region was used in the present study to confirm the isolates as *R. homothallicus*. Chakrabarti et al. confirmed the identity of the isolates in their study using sequencing of the D1/D2 region of the 28S rDNA [15].

Treatment of mucormycosis consists of medical management with appropriate antifungal therapy, surgical intervention when needed and correction of the underlying risk factors [3]. Amphotericin B in the conventional form or the liposomal form is the drug of choice for most mucormycosis. Though it is a first-line drug, Amphotericin B is not effective if the patient presents late during the course of the disease or has inoperable or disseminated mucormycosis [2]. In the present case series, two cases were treated with liposomal amphotericin B and one with conventional amphotericin B.

Clinical suspicion of mucormycosis warrants prompt confirmation using reliable diagnostic measures. Institution of appropriate treatment with control of underlying risk factors is essential to reduce morbidity and mortality by the offending Mucorales.

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