Cavitary Pulmonary Zygomycosis Caused by *Rhizopus homothallicus*

Short title: Zygomycosis due to *Rhizopus homothallicus*

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We report the first two proven cases of cavitary pulmonary zygomycosis caused by Rhizopus homothallicus. Diagnosis in each case was based on histology, culture of the causal agent, and nucleotide sequence of the D1/D2 region of 28S rDNA.

**Key words:** pulmonary zygomycosis, Rhizopus homothallicus
Case 1: A 47-year-old male with a history of type II diabetes mellitus for 13 years developed diabetic nephropathy leading to the end stage renal disease. He had undergone renal transplantation and was on triple drug immunosuppression (cyclosporine, azathioprine, and prednisolone). One month post-transplant, he developed intermittent fever with chills and pleuritic chest pain. Chest X-ray revealed cavitary lesion in the right upper lobe with bilateral nodular infiltrates for which he was treated with primary drug regimen of anti-tuberculous therapy (ATT), though multiple sputum examinations were negative for acid-fast bacilli. The patient did not respond to the ATT and was referred to our institute (Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India). At our institute, contrast enhanced computed tomography (CECT) of thorax revealed thick, smooth-walled, cavitary lesion on posterior segment of right upper lobe and multiple nodules in both lungs (Fig. 1). No pleural effusion was noticed. Echocardiography ruled out endocarditis. His blood sugar ranged between 153-226 mg/dL. While he was at the hospital he was treated with human insulin therapy. ATT was continued along with vancomycin and tazobactam. An ultrasound–guided fine needle aspiration from the cavitary lesion of the lung was performed. Aspirated fluid was examined by direct microscopic examination of calcofluor white-stained mounts of the aspirated fluid. A part of the fluid was cultured on Sabouraud dextrose agar (SDA) and brain heart infusion (BHI) agar (HiMedia, Mumbai, India). Direct microscopy of the aspirated fluid did not reveal any fungal elements nor the cultures yield any fungal colonies until the end of 4 weeks incubation. As the patient did not improve until 20th day of hospital stay, open lung biopsy was performed. It revealed pus in the pleural
cavity with large cavitary lesion in right upper lobe. No apparent mass was noticed. Wedge-shaped biopsy was taken from the site of the lesion. Tissue slides stained by haemotoxylin and eosin, and periodic acid Schiff’s procedures showed dense acute inflammatory infiltrates across the interstitial septa, a dense fibrosis surrounding the alveoli, extensive necrosis with nuclear debris and broad, aseptate, ribbon-like, hyphae (Fig.2). The causal fungus was observed invading the vessel wall. Culture of the biopsy tissue on SDA (HiMedia, Mumbai, India) grew a fast-growing, floccose white colony turning grayish in color. Microscopic examination of the colony showed broad aseptate hyphae with lateral not well-developed sporangiophores bearing globose sporangia containing a small number of sporangiospores. In addition, a large number of golden-brown zygospores with stellate walls and with unequal suspensor cells were observed. The isolate was presumptively identified as *Rhizopus homothallicus* Hesseltine and Ellis (8). Liposomal amphotericin B (Fungisome®, Lifecare innovations, India) was given at a dose of 1.5 mg/kg of body weight/day. Follow up after cumulative dose of 5.0 gm of liposomal amphotericin B therapy showed striking improvement of the patient both clinically and on radiological investigations. No relapse or recurrence of the infection was noticed until 5 months of follow-up after recovery.

**Case 2:** A 70-yr-old male presented at the King George Medical University Hospital, Lucknow, India, with a history of fever, cough, chest pain mucopurulent expectoration, and recurrent haemoptysis of 25 days duration. On general examination, he had signs of glossitis and stomatitis. Examination of respiratory system revealed bronchial breath sound over left mammary area and rest of the physical examination was unremarkable. Chest radiograph revealed the presence of air space consolidation with eccentric
cavitation in mid zone of the left lung. Computed tomography of thorax revealed a large thick-walled cavity in the left upper lobe abutting chest wall and encroaching towards arch of aorta. On routine investigations, uncontrolled blood sugar levels (range 232-360 mg/dL) were noted. Sputum examination did not reveal acid-fast bacilli. The patient was treated with oral antibiotics (amoxicillin–clavulainic acid 625 mg twice daily and clindamycin 150 mg four times a day for 2 weeks). He did not show clinical or radiological improvement. The patient refused to undergo bronchoscopy. Transthoracic needle aspiration from the left cavitary lesion revealed ribbon-like, broad, coenocytic hyphae on direct KOH examination as well as smears stained by periodic acid Schiff’s stain and Gomori’s methenamine silver stain procedures. Fungal culture of the aspirate on SDA (HiMedia, Mumbai, India) grew white, cottony, colonies. Direct examination of a teased mount of the colony stained by lactophenol cotton blue showed numerous golden-brown globose, spiny, zygospores with suspensor cells. The isolate was tentatively identified as *R. homothallicus*. The patient was treated with injectable insulin for glycemic control, and intravenous conventional amphotericin B (50 mg/day) for 15 days. After a cumulative dose of 750 mg. of amphotericin B, the patient developed acute renal failure. After the withdrawal of amphotericin B, his renal function improved rapidly. However, he refused subsequent treatment with amphotericin B and left the hospital against medical advice. He returned after 3 months with massive haemoptysis and succumbed to his illness rapidly before any treatment with antifungal agents could be initiated.

Colonies of both isolates on SDA were fast growing, white, floccose devoid of pigmentation on the reverse of the colonies. Within 10-14 days of incubation at 28°C-
30°C, colonies turned grayish. Slide culture mounts of the isolate from Case 1 stained by lactophenol cotton blue [PGIMER MCCL (Mycology Culture Collection laboratory, No. 710076)] on SDA incubated at 30°C for 10 days showed broad, hyaline, aseptate, branching hyphae producing very few sporangiophores opposite poorly developed rhizoids. Sporangiophores measured 5-27µm in diameter and 50-150µm in length bearing globose to sub-globose sporangia measuring 50-150µm in diameter. Sporangiospores were angular-globose, grayish, 3.5-5.0µm in length and 4.0-6.5µm in width. The striking feature was abundant number of homothallic, thick-walled zygospores, reddish brown in color measuring 40-100µm in diameter including stellate spines. Suspensor cells were uneven in size, the larger being globose (Fig.3).

The isolate from Case 2 (MCCL 710099) was studied at the CDC. Slide cultures on potato dextrose agar and malt extract agar after 2 weeks at 25°C did not show any fertile sporangia containing sporangiospores. Sporangiospores were poorly developed opposite poorly developed rhizoids in tufts of 2-5 at several locations. Due to lack of any sporulation, agar blocks (2 x 2 cm) with mycelial growth from the culture plates were transferred aseptically to a plate containing 20ml of sterilized distilled water to which 3-5 drops of 15% filter sterilized yeast extract solution was added. After 5 days of incubation at 37°C, examination of growth over the surface of water showed abundant globose, golden brown, spiny zygospores supported by uneven size suspensor cells. Both isolates were thermotolerant and grew at 46°-48°C. Both isolates were identified as *Rhizopus homothallicus* Hesseltine and Ellis (8).

The identity of both isolates was further confirmed by nucleotide sequence of 28S ribosomal region. Whole cell DNA from the isolates was extracted using a slightly
modified protocol of small-scale fungal DNA extraction method described by Lee and
Taylor (10). Briefly, pure cultures of the isolates were grown in Sabouraud dextrose broth
(HiMedia, Mumbai, India) and incubated at 37°C on a rotary shaker (HT Infors, Germany) at 120 rpm for 3 to 5 days. The mycelial mat (0.4-0.5 gm) was prepared and crushed to fine powder in a pestle and mortar in the presence of liquid nitrogen and lysis buffer. The DNA was extracted using standard phenol:chloroform (25:24) extraction method. DNA precipitation was carried out using 2 volume of chilled absolute alcohol and 1/5th volume of 10M-ammonium acetate, followed by washing with 70% alcohol. The DNA pellet was dissolved in 100 µl of TE buffer. DNA preparations were stored at -20°C till use. PCR was performed in a reaction mixture of 10µl containing 2mM MgCl₂, 200µM of each dNTP (Bangalore Genie, Bangalore, India), 0.25µm of each primer (Integrated DNA Technologies, Inc., Coralville, Iowa), NL1 (GCATATCAATAAGCGGAGGAAAAG) and NL4 (GGTCCGTGTTTCAAGACGG), 0.25U of Taq polymerase (Bangalore, Genie), and 10ng of fungal genomic DNA. The amplification reactions were performed in Eppendorff Mastercycler (Hamburg, Germany). The amplification was performed for 36 PCR cycles with annealing at 52°C, extension at 72°C for 2 min, and denaturation at 94°C for 1min. PCR products were purified using gel extraction kit (Qiagen Hilden, Germany), and both the strands were sequenced by the Big Dye terminator cycle sequencing ready reaction kit, version 3.1 (Applied Biosystems, Foster City, CA) with the primers NL1 and NL4. The reaction products were analyzed on Genetic Analyzer 3130 (Applied Biosystems). Basic local alignment search tool (BLAST) was used to compare the sequences obtained with those in the GenBank database and to see the similarity of both isolates. The sequences of both
isolates gave 99% identity with each other and 98% identity with the ex-type strain of *Rhizopus homothallicus* (AB 250198, NRRL 2538 = CBS 336.62). Sequence data of the Indian isolates (MCCL 71006 and MCCL 710099) were submitted to the GenBank (Accession No. EU 128745 and EU 491016). The two Indian isolates have been deposited in the CBS Fungal Biodiversity Centre, Utrecht, The Netherlands, with the following accession numbers: (MCCL 71006 = CBS 125071 and MCCL 710099 = CBS 125072).

The antifungal susceptibility testing of both isolates was performed by micro-dilution broth technique following the protocol of Clinical Laboratory Standard Institute (CLSI) document M-38A (5). The minimum inhibitory concentration (MIC) of both isolates were similar: amphotericin B – 0.5 µg/ml; flucytosine - >64.0 µg/ml; fluconazole – 64.0 µg/ml; itraconazole - >16.0 µg/ml; voriconazole – 4.0 µg/ml; and caspofungin – 16 µg/ml.

Among the different agents of zygomycosis, *Rhizopus* spp. are the most commonly implicated agents causing human infections, and *R. oryzae* is the most predominant species implicated in 90% of cases of invasive zygomycosis (3,4,17). The other *Rhizopus* spp. less commonly reported as causal agents are *R. microsporus* (16), *R. azygosporus* (6), *R. schipperae* (2), and *R. stolonifer* (7). To our knowledge, the present report describes the first two cases of invasive zygomycosis caused by *R. homothallicus*. Hesseltine and Ellis described *R. homothallicus* in 1961 based on ex-type (NRRL 2538) isolated from a soil sample from Guatemala in 1956 (8). Subsequently, the species had been isolated in India from soil samples from several areas, from dung, stored grains of
Triticum sp. (http://www.cabri.org/HyperCat/fun/all102646.htm). When isolated from soil or other environmental sources, *R. homothallicus* closely resembles *R. microsporus* in general morphology especially asexual sporangiophores, sporangia, sporangiospores, and maximum growth temperatures (19). However, strains maintained under laboratory conditions often lose their sporulation ability including ability to form zygospores. According to Schipper and Stalpers, the ex-type strain of *R. homothallicus* (NRRL 2538 = CBS 336.62) no longer produces zygospores (19). In 1970, Scholer attempted to produce experimental infection in mice using *R. homothallicus* but was not successful. The reason of failure was considered to be due to insufficient number of sporangiospores in the inoculum, and failure to inject large size zygospores intravenously (20).

Schipper (18) classified *Rhizopus* spp. into three groups; namely, *R. stolonifer* group, *R. oryzae* group, and *R. microsporus* group based on phenotypic characters and maximum growth temperatures (18,19). Recent studies by Abe et al. (1) based molecular phylogeny of *Rhizopus* spp. have also concurred with Schipper’s treatment of *Rhizopus* spp. groups.

Earlier observations by Scholer (20) and recent observations by Jennessen (9) have stressed that in *R. homothallicus* rhizoids, sporangiophores, sporangia, and sporangiospores are often poorly developed. We also observed similar findings in our two isolates. Production of abundant zygospores was the main phenotypic character that was helpful in the identification of isolate from Case 1. The isolate from Case 2 failed to produce zygospores when grown on routinely used potato dextrose agar and malt extract agar. A low nutritional medium had to be used to induce zygospore production (14). *Rhizopus homothallicus* can also be confused with another homothallic species, namely,
R. sexualis, which also produces abundant zygospores. However, R. homothallicus grows at temperatures as high as 46°C-48°C, while R. sexualis does not grow at 37°C.

Given the limitation of phenotypic identification methods, ribosomal DNA (rDNA) based gene sequences have been used extensively for molecular identification of fungi including zygomycetes (1, 11, 23). The rDNA comprise of small subunit gene (18s), large subunit gene (28s), and internal transcribed regions (ITS1 and ITS2). The ITS region is generally used for the species identification of fungi as the sequences can be aligned with confidence between closely related taxa. To obtain similar resolution with 18s and 28s genes, a large portion of the molecule must be sequenced. In the present study, many attempts to sequence the ITS region of rDNA failed (results not shown).

Hence the sequencing of the D1/D2 region of the 28s rDNA was performed. The failure to obtain pure sequence of the ITS region may be attributed to the homothallic nature of the fungal species under study, which led to multiple distinct ITS regions in a single strain. The presence of multiple bands did not allow proper analysis of the sequences. The presence of multiple distinct ITS regions had been described earlier with another homothallic Rhizopus species, R. sexualis. Therefore, sequencing of D1/D2 region of 28s rDNA may be more useful and easy method for the identification of homothallic R. homothallicus (1, 11, 23).

Pulmonary zygomycosis is considered second in frequency after rhino-orbital-cerebral type among different categories of zygomycosis and has rarely been reported without any predisposing factor. Cough, fever, and pleuritic chest pain are the common presenting symptoms in patients with pulmonary zygomycosis (22). Pulmonary zygomycosis may have a wide variety of lesions including isolated solitary nodule, lobar...
involvement, cavitary or disseminated lesions (13, 15, 22). Pulmonary consolidation, cavitation or an effusion is less frequently seen (13, 22). Both patients in the present report had cavitary lesions. Tedder et al. (22) in a review of 156 cases of pulmonary zygomycosis observed only 6.0% had radiographic findings of cavitation of the lung. In our earlier two series of reports on zygomycosis from PGIMER, 25 patients had pulmonary zygomycosis and none had cavitory lesions (3,4), though contrasting claim of approximately 40% cavitary lesions among patients with pulmonary zygomycosis had been made (12). However, it is not clear whether the type of lesion depends on virulence of the causal agent or host immune status or both. It was suggested that such cavities represent liquefaction of pulmonary infarcts (13). The correlation between the type of fungi of Mucorales isolated from the pulmonary lesion and the type of lesion has never been established. Interestingly, few species like R. stolonifer (7) and Cunninghamella bertholletiae (24) have been isolated from patients with cavitary lesions.

Haemoptysis in patients with zygomycosis may be fatal and may occur due to erosion of the cavitary lesion into bronchus (23). The patient from Case 2 had similar fate. Sputum or broncho-alveolar lavage analysis, though frequently employed, rarely leads to confirmation of diagnosis. Procedures such as open lung biopsy, surgical extirpation, transthoracic needle aspiration provide better samples for diagnosis (22). In the present two cases, invasive procedures helped in the definitive diagnosis.

Without prompt therapeutic management, invasive zygomycosis invariably proves fatal. Aggressive surgical treatment combined with appropriate medical therapy as well as controlling predisposing factors are of vital importance to treat such cases (3). Amphotericin B is the first line of drug of choice for most of the cases of zygomycosis.
both cases presented here, patients were treated with amphotericin B using either conventional or liposomal formulation. The patient from Case 1 responded well to the therapy. The second patient succumbed to the infection possibly due to inadequate treatment. Both isolates of *R. homothallicus* had MICs of 0.5µg/ml against amphotericin B. The MIC patterns observed with the two isolates of *R. homothallicus* were consistent with those reported for other *Rhizopus* species (21).

**Nucleotide sequence accession numbers:** Nucleotide sequences data of 28s rDNA region of both isolates were submitted to the GenBank (Accession No. EU128745 – MCCL 710076, EU491016 – MCCL 710099)

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**Conflict of interest:** None of the authors have an association that might pose a conflict of interest in relevance with the manuscript.
REFERENCES


Explanation of Figures

Figure 1: Axial contrast enhanced CT sections of the chest - mediastinal window (Fig. 1A) and lung window (Fig. 1B), revealing a thick-walled cavity in the posterior segment of right upper lobe (white arrow) with multiple irregular septations within it. Also seen are a few lymph nodes in the pretracheal location.

Figure 2: Periodic acid Schiff’s stained lung tissue section showing dense, acute inflammatory infiltrates, fibrosis, necrosis, and aseptate, hyphal elements (Case 1) X 100

Figure 3: Lactophenol cotton blue mount of *R. homothallicus* (MCCL 710076) showing numerous golden-brown, globose zygospores with stellate spines X 200 (insetX400)
Figure 2
Figure 3