**Rhinocladiella mackenziei** as an Emerging Cause of Cerebral Phaeohyphomycosis in Pakistan: A Case Series

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Six cases of *Rhinocladiella mackenziei* cerebral phaeohyphomycosis are being reported for the first time in Pakistan. Identification was confirmed by DNA sequencing (isolates and fixed tissue). Diabetes, head trauma, immunosuppressive treatment, and postpartum state were present in 4 cases. Two survivals and 3 fatalities occurred, with 1 patient lost to follow-up.

*Rhinocladiella mackenziei* (formerly *Ramichloridium mackenziei*), a melanized neurotropic fungus, is one of the commonly reported agents of cerebral phaeohyphomycosis[1]. The majority of *R. mackenziei* infections occur in immunocompetent individuals[2]; however, it has been reported in patients with diabetes, hematological malignancies, systemic lupus erythematosus, chronic liver disease, and renal transplantation, as well as after chemotherapy[1–3]. Cerebral infections with *R. mackenziei* have been associated with very low survival rates. *R. mackenziei* grows slowly in culture media, and its accurate diagnosis requires expertise and often consultation with reference laboratories. Confirmed diagnosis of the infection requires visualization of pigmented fungal elements along with isolation of the fungus from biopsy sample or aspirated pus from brain lesions[4]. Central nervous system infections due to *R. mackenziei* have been exclusively reported from the Middle East, except for cases recently reported from India and Afghanistan [5–6]. Infection with *R. mackenziei* has never been previously reported from Pakistan. This case series describes 6 cases of cerebral *R. mackenziei* infection from arid regions of Baluchistan and Sind.

**CASES**

Details of the cases are described in Table 1. All cases underwent surgical excision of the lesion, followed by antifungal agent, used singly or in combination (Table 1).

**LABORATORY IDENTIFICATION**

Material was mixed with 10% KOH and was visualized at 10X and 40X magnification. Lactophenol cotton blue staining was used to confirm septations and pigment in doubtful cases. All samples revealed inflammation with moderate to numerous darkly pigmented hyphae. Several toluroid and moniliform hyphae were also visualized, but no yeast forms were noted (Figure 1). Specimens were inoculated on sheep blood agar, Saboraud’s dextrose agar, potato dextrose agar, and Mycosel and BiGGY agar. Plates were incubated at 27°C and 37°C and were observed daily. In the laboratory, most of the isolates were negative at 4 weeks and started to grow initially as black discoloration of media. Unless the laboratory has a high index of suspicion, the culture plates may be discarded at 4 weeks, falsely reporting these as fungal culture negative. Slide cultures were prepared on malt extract agar or tap water agar. *Rhinocladiella*-type sporulation (“Mickey Mouse” appearance) was seen in all isolates, with no additional sporulation types even after prolonged incubation.

Four isolates (cases 1–4) were sent for confirmation to the Centers for Disease Control and Prevention (CDC), where the D1/D2 (28S) ribosomal DNA region was sequenced [7]. Genomic DNA was extracted using Omnimixer and DNeasy Tissue kit (Qiagen, Valencia, California), and amplification was performed under conditions previously described[7] by using PfX DNA polymerase (Invitrogen Tech-Line). Amplicons were purified with the ExoSAP-IT PCR purification kit (USB), and products were directly sequenced using the same primers as for PCR amplification, as described elsewhere [8]. Sequences were edited using Sequencer, version 4.9 (GeneCodes), and were compared with the sequences deposited in GenBank using the BLAST algorithm. DNA from all 4 isolates showed 99%
<table>
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<tr>
<th>Case</th>
<th>Age and Gender</th>
<th>Residence and Occupation</th>
<th>Comorbidities</th>
<th>Clinical Presentation</th>
<th>Radiologic Findings</th>
<th>Histopathology</th>
<th>D1/D2 28S rDNA Sequencing</th>
<th>Therapy</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>45, male</td>
<td>Balochistan, shopkeeper</td>
<td>Diabetic, hypertensive, paraplegic with bed sores</td>
<td>Fever for one month, slurred speech, dysphagia, right hemiparesis</td>
<td>MRI: ring enhancing lesions in centrum semi-ovale, left middle peduncle, and right periventricular region</td>
<td>Numerous pigmented septate hyphae with extensive necrosis</td>
<td>Isolate sequencing positive for <em>Rhinocladiella mackenziei</em>; tissue sequencing not performed</td>
<td>Surgical evacuation twice; Amphotericin B deoxycholate 1mg/kg/day and itraconazole 200 mg BD for 6 weeks</td>
<td>Death at one month</td>
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<tr>
<td>2</td>
<td>30, male</td>
<td>Sindh, woodcutter</td>
<td>Head injury with tree bark 2 years prior to presentation</td>
<td>Progressive right arm weakness, fever, and vomiting</td>
<td>MRI: frontoparietal lesion</td>
<td>Moderate pigmented septate hyphae with toruloid forms; granulomatous inflammation</td>
<td>Isolate sequencing positive for <em>Rhinocladiella mackenziei</em>; tissue sequencing negative for fungi</td>
<td>Frontoparietal craniotomy and evacuation; Amphotericin B deoxycholate 1mg/kg/day and itraconazole 200 mg BD given duration unknown</td>
<td>Alive at 10 months, worsening focal deficits. No contact after that</td>
</tr>
<tr>
<td>3</td>
<td>45, male</td>
<td>Unknown</td>
<td>None known</td>
<td>Fever, vomiting, and focal deficits</td>
<td>Not known</td>
<td>Pigmented septate moniliform hyphae; granulomatous inflammation</td>
<td>Isolate sequencing positive for <em>Rhinocladiella mackenziei</em>; tissue sequencing negative for fungi</td>
<td>Surgical evacuation; Amphotericin B deoxycholate 1mg/kg/day and itraconazole 200 mg BD given duration unknown</td>
<td>Lost to follow-up</td>
</tr>
<tr>
<td>4</td>
<td>20, female</td>
<td>Balochistan, housewife</td>
<td>20 days post-partum</td>
<td>Fever, headache, vomiting, and right-sided weakness; had 2 seizures during therapy</td>
<td>MRI: left posterior frontal cortex lesion</td>
<td>Numerous pigmented toruloid hyphae; granulomatous inflammation</td>
<td>Isolate and tissue sequencing positive for <em>Rhinocladiella mackenziei</em></td>
<td>Neuronavigation-guided craniotomy and aspiration; Amphotericin B deoxycholate 1mg/kg/day and itraconazole 200 mg BD for 4 weeks, then itraconazole alone following this</td>
<td>Alive at 12 months; therapy continued, lesions resolved on imaging, resolution of focal deficits</td>
</tr>
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</table>
homology with GenBank accession number EU041866.1 R. mackenziei (CBS 368.92). One isolate also gave 100% homology with GenBank accession number FJ427212.1 R. mackenziei and with GenBank accession number AF050288.1 R. mackenziei.

Sequencing of the internal transcribed spacer (ITS) regions was attempted, but no amplification was obtained.

Formalin-fixed paraffin-embedded blocks from cases 2–6 (5 blocks) were used for direct extraction of fungal DNA. The block from case 1 could not be located for testing. Fungal DNA was extracted and amplified, and the ITS 2 region was sequenced from 3 of the 5 blocks (cases 4–6) according to methods previously described [9]. The recovered DNA from blocks 4–6 (285 nucleotides each) showed a 99% match to multiple R. mackenziei sequences in GenBank. Fungal DNA could not be recovered from 2 of the blocks (cases 2 and 3), although the human globin DNA control could be amplified.

**DISCUSSION**

This series represents the first reported cases of cerebral phaeohyphomycoses due to R. mackenziei from Pakistan. At this time the literature describes about 22 reports, all in residents of Middle Eastern countries, including Saudi Arabia, Syria, and Kuwait [1–2, 4]. The reason for this endemicity has been related to the hot and arid climate in these countries. Recently cases of cerebral abscess due to R. mackenziei have been reported from India and Afghanistan, regions on non-endemicity for this mold [5–6].

Diagnosis in our cases was established using phenotypic characteristics in Karachi and was confirmed using both morphology and molecular methods at the CDC. DNA matching R. mackenziei sequences was recovered from each of the 6 cases, either from an isolate or a paraffin block. Sequencing results of 4 isolates were consistent with conventional identification. Two isolates from cases in the latter part of the study could not be sent for molecular identification. Identification of R. mackenziei from Formalin-fixed paraffin-embedded tissue blocks was positive in 3 of 5 cases, including the 2 cases in which isolates were not available. The negative DNA results in 2 of the paraffin blocks despite the presence of fungal elements on histopathology could be explained by DNA cross-linking due to the type of fixative, storage time, temperature variability, or presence of PCR inhibitors, although the human control could be amplified from the material [9].

In our series, all the patients (20–53 years of age and 5 of 6 were male). They had no history of travel outside the country and resided in Balochistan (3 patients) and inland Sindh (2 patients), both hot and dry areas consistent with the climate of the known endemic region where R. mackenziei is endemic. Cerebral R. mackenziei infections may be a result of either hematogenous or direct spread from accidental introduction of spores at contiguous sites [10]. We were unable to ascertain the route of infection in these cases, although in the

<table>
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<tr>
<th>Case</th>
<th>Age</th>
<th>Sex</th>
<th>Occupation</th>
<th>Previous history</th>
<th>Febrile symptoms</th>
<th>Lesion characteristics</th>
<th>Tissue sequencing</th>
<th>Surgical excision</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>53, male</td>
<td>Balochistan, forest officer</td>
<td>Fever, headache, vomiting, and left-sided weakness</td>
<td>Pigmented moniliform hyphae, granulomatous inflammation</td>
<td>Tissue sequencing positive for <em>Rhinocladiella mackenziei</em>, isolate not sequenced</td>
<td>Surgical excision of lesion; Amphotericin B deoxycholate 1 mg/kg/day</td>
<td>Death at 2 months</td>
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</tr>
<tr>
<td>6</td>
<td>75, male</td>
<td>Sindh, landowner/farmer</td>
<td>Fever, left-sided hemiplegia since 2 months</td>
<td>Pigmented moniliform hyphae with granulomatous inflammation</td>
<td>Tissue sequencing positive for <em>Rhinocladiella mackenziei</em>; isolate not sequenced</td>
<td>Craniotomy and evacuation; Itraconazole 200 mg BD duration unknown</td>
<td>Alive at 2 months; focal deficits and brain lesion resolving on imaging; rehospitalized for urinary tract infection at 3 months, complicated hospital stay, and expired at 4 months</td>
<td></td>
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</table>
patient with a history of head trauma there is a high possibility of direct inoculation of the spores as a result of skull injury.

Only 1 patient, who was post–renal transplantation, had a clear history of immunosuppression. Of the rest no clear risk factors could be ascertained.

Cerebral phaeohyphomycoses with \textit{R. mackenziei} is associated with poor outcome, and mortality is reported to be almost 100% despite surgical intervention and antifungal therapy\cite{2,3,4}. Of the 6 patients in our series, 3 did not survive (2 had significant comorbidities), and 1 patient was lost to follow-up. Case 6 did show shrinking lesions on CT but died of complicated obstructive uropathy and urosepsis at the fourth month of craniotomy. The remaining 2 patients were both alive at the 6-month follow-up, though one of these patients subsequently discontinued his antifungal therapy without advice and had developed symptoms again at 10 months. He was later lost to follow-up. The other patient at 15-month follow-up was well, with no evidence of disease progression. The survival rate in our series seems apparently higher than that reported previously; however, the surviving patients still require further follow-up to document cure. There has been only one previously reported case of successful treatment of \textit{R. mackenziei} brain abscess\cite{11}. That patient showed improvement after switching to therapy from itraconazole to posaconazole. Posaconazole and, recently, isavuconazole have been reported to be effective in vitro against this agent\cite{12}. However, these drugs are not freely available in developing countries, and a recent report of treatment failure with posaconazole is also of concern\cite{3}. We recognize that all of our patients received suboptimal therapy; the cost of newer antifungals was a great limitation. Due to resource constraints one of our patients was not even able to continue itraconazole (case 2, the woodcutter) and went back to his remote village in Sind and declined follow-up. The patients who could afford voriconazole (cases 4 and 5) both responded favorably to the available itraconazole radiologically as well as clinically. Case 5 was also advised to take voriconazole, but before the drug could be made available (being found only at one center in Karachi and that in oral form), the patient deteriorated and expired.

The emergence of \textit{R. mackenziei} from Pakistan, previously recognized as a country of nonendemicity for this fungus, is alarming. High mortality and the nonavailability and high cost of effective antifungal agents are our major concerns.

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Potential conflicts of interest. All authors: no conflicts.

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