

# 57 *Phialemonium*

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## 57.1 INTRODUCTION

### 57.1.1 CLASSIFICATION, MORPHOLOGY, AND BIOLOGY

The genus *Phialemonium* consists of dematiaceous (i.e., dark-walled) fungi belonging to the mitosporic Ascomycota group, class Sordariomycetes, subphylum Pezizomycotina, phylum Ascomycota, kingdom Fungi. Currently, the genus comprises two recognized species (*Phialemonium curvatum* and *Phialemonium obovatum*) and nine unassigned species. A former species in the genus *Phialemonium dimorphosporum* is synonymized with *P. curvatum* [1]. Anamorphs of the genus are found in Cephalothecaceae.

First created in 1983 by Gams and McGinnis to cover fungal isolates with morphological features intermediate between *Acremonium* (which is hyaline or nonpigmented) and *Phialophora* (which is pigmented), interestingly, *Phialemonium* appears pale in culture and the innate hyphal pigmentation is observed in tissue only by histological stains with specificity for melanin (e.g., the Fontana-Masson silver stain) [2].

*Phialemonium* colonies grow rapidly, reaching a diameter of 25–30 mm in 7 days on Sabouraud dextrose agar (SDA). Initially, colonies appear flat, moist, and white, and later become yellowish, with yellow-green pigments diffusing into media (in the case of *P. obovatum*). Conidiophores are hyaline and resemble hyphae; intercalary and distinct septate phialides (10–16  $\mu\text{m}$  in length) without collarettes arise from hyphae; conidia (2–3  $\mu\text{m}$  by 4–5  $\mu\text{m}$  in size) are one-celled and hyaline, smooth walled, aggregating into slimy obovate or allantoid balls [3].

*P. curvatum* conidia are subglobose to ellipsoidal or curved. Phialoconidia formed in tapering phialides are located in short branches of the superficial and submersed hyphae. These short prolongations are cylindrical and thin and lack a collarette. Adelophialides and some phialides

are often without a basal septum. Conidia are unicellular, hyaline, and allantoid. On the other hand, *P. obovatum* produces a distinctive pale green pigment in culture, in contrast to other medically important dematiaceous fungi (which are olive green or brown to grey-black). *P. obovatum* conidia are obovoid or narrowest at the base.

*Phialemonium* species are saprophytic in nature and represent a rare cause of invasive mold infections in humans, especially immunocompromised hosts and transplant recipients. *Phialemonium* spp. have the ability to sporulate within the matrix of the agar medium, without the requirement of an air space above the hyphae, which is also noted in hyaline filamentous molds such as *Acremonium* and *Fusarium*. The production of unicellular yeast-like forms or propagulation in vivo by *Acremonium*, *Fusarium*, and *Phialemonium* enables their ready circulation in the bloodstream (resulting in fungemia) in comparison with hyphal structures of angioinvasive fungi such as *Aspergillus* and *Mucor*. In addition, *Phialemonium* species are able to grow in culture at temperatures as high as 40°C, suggesting their potential for infection of the central nervous system.

### 57.1.2 CLINICAL FEATURES AND PATHOGENESIS

Once thought to possess low pathogenic potential, the dematiaceous (or melanized) fungi have increasingly become common causes of severe and disseminated phaeohyphomycosis, affecting cutaneous and subcutaneous tissues, the ocular region, frontal and maxillary sinuses, lungs, bones, and the nervous system [4]. The genus *Phialemonium* is considered as a pheoid fungus, which may cause the lesions observed in phaeohyphomycosis and hyalohyphomycosis [5–8]. Predisposing factors for fungal infections are antibiotic therapy, post-transplant immunosuppressive therapy, and HIV infections. Another important risk factor for *Phialemonium*

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infection is the use of arteriovenous (AV) grafts or fistulas for hemodialysis, as many infected patients had AV grafts or fistulas rather than catheters. It is possible that implanted synthetic grafts or surgically created fistulas may act as a nidus for subclinical *Phialemonium* infection, allowing small inocula to produce overt infection more readily in a graft or fistula than in a catheter. Additionally, prior to dialysis access, thorough skin antisepsis is required for grafts and fistulas. Any suboptimal skin preparation may facilitate viable molds on the skin to be inoculated into the bloodstream or the AV graft. In addition, the use of prefilled syringes may also act as the source of *P. curvatum* infection [9].

*P. curvatum* infections have been reported in patients with bone marrow transplantation, and hemodialysis patients, who developed overwhelming infection associated with fungemia and endocarditis [9–15]. A case of cutaneous infection caused by *P. curvatum* was reported in a patient with multiple myeloma who underwent bone marrow transplantation. Two months after transplantation, the patient developed purplish and painful nodular lesions on the right ankle. Lesion material contained septate hyphae and some apparently hyaline dilated cells. On potato agar, initially white colony later became chraceous with whitish borders forming moist and plane colonies. The colony became grayish on Sabouraud agar with sparse white mycelium. The fungus was identified as *P. curvatum* on the basis of its unicellular, hyaline, cylindrical, and allantoid conidia, in addition to other characteristic macroscopic and microscopic characteristics [16].

In the four cases of *P. curvatum* infections reported by Proia et al. [11], the first involved a 35 year-old man with chronic renal failure. The patient presented right ocular pain, swelling, and vision loss, 1 year after cadaveric renal transplantation surgery and oral prednisone and cyclosporine treatment through hemodialysis via AV fistula. Physical inspection of the right eye revealed periorbital edema, scleral injection with hemorrhagic foci, and vitreal clouding. Gram staining of vitreal aspiration showed both hyphal and yeast-like forms. A transesophageal echocardiogram (TEE) uncovered large vegetations on both mitral and aortic valves. Primary blood cultures on BacT/ALERT aerobic and anaerobic bottles (bioMérieux) at 30°C and 35°C became positive after 4 days; subculture showed heavy growth of *P. curvatum* mold after 3 days. The patient underwent combined aortic and mitral valve replacement and right eye vitrectomy. Gram staining of mitral valve tissue revealed septate hyphae, and cultures of mitral and aortic valve tissue specimens grew *P. curvatum*. One day after the operation, the patient became febrile and hypotensive and developed acute congestive heart failure and disseminated intravascular coagulation and then died. Postmortem examination revealed cardiomegaly, marked left ventricular dilatation, splenomegaly, and a large splenic abscess. A large “vegetation” had overgrown the prosthetic mitral valve and had invaded through the left ventricular wall.

The second case concerns a 66 year-old man who had undergone long-term hemodialysis after AV graft. The patient presented a soft systolic murmur over the cardiac

apex. Three blood cultures and a graft tissue specimen culture grew *P. curvatum*. TEE showed a small, calcified vegetation on the anterior leaflet of the mitral valve that extended into the left atrium, with mild mitral regurgitation. The third case involves a 70 year-old woman undergoing long-term hemodialysis. The patient had cerebrovascular accident, type II diabetes mellitus, and peripheral vascular disease. A *Phialemonium* species grew on culture of a graft tissue specimen. The fourth patient involves a 77 year-old man with type II diabetes mellitus and chronic renal failure requiring hemodialysis. Ultrasonography of the AV graft revealed thrombosis; histopathologic examination of resected graft material showed inflammatory cells and numerous fungal elements, and culture of the AV graft and blood grew *Phialemonium* species [11].

*P. obovatum* has been linked to cases of endocarditis, fungemia, and osteomyelitis [17–19]. A case of native valve endocarditis caused by *P. obovatum* in a premature neonate was reported [20]. A 4.5 month-old infant with fatal *P. obovatum* infection of thermal burn wounds, viable tissue, and blood vessels, with dissemination to the spleen. *P. obovatum* osteomyelitis has been observed in a previously healthy 41 year-old man following discography. A case of *P. curvatum* fungemia in a patient with chronic myelogenous leukemia was reported. The patient developed graft-versus-host disease of the skin and liver with fever and severe diarrhea 6 months after peripheral bone marrow transplantation. Biopsies of the stomach, colon, and rectum showed granulomatous inflammation with marked crypt distortion simulating Crohn disease. A lung wedge biopsy of the lesion revealed septate branching hyphae (4–5 µm in diameter) with terminal globular structures (10 µm in diameter). Blood cultures grew a pure isolate of *P. obovatum* [21].

The in vitro susceptibility of *Phialemonium* indicated a more consistent level of activity for voriconazole and posaconazole. Treatment with the new triazoles is associated with improved survival [8]. Caspofungin combined with amphotericin B deoxycholate may have an additive effect.

### 57.1.3 DIAGNOSIS

Although *Phialemonium* is considered as a dematiaceous fungus, direct microscopic examination of the material obtained from its lesions often shows hyaline septate hyphae and its culture also appears pale. Since other phaeohyphomycotic agents such as *A. alternata*, *Bipolaris spicifera*, *Exophiala jeanselmei*, or *E. spinifera* also appear hyaline, they may complicate the diagnosis of *Phialemonium* infection. The differentiation of *Phialemonium* from other phaeohyphomycotic agents relies on the detection of the innate hyphal pigmentation in *Phialemonium* with the Fontana-Masson silver stain that binds specifically to melanin.

Microscopically, the distinctive features of *P. curvatum* isolates include the presence of setose and cupulate sporodochium, conidium formation from branched conidiophores, conidium production on typical tapering adelophialide lacking a basal septum, and hyphae with lateral and intercalary

phialidic conidiogenous cells, with typical clusters of conidia aggregating in slimy heads [9]. By contrast, *Phialemonium obovatum* isolates show obovate straight conidia (3.5–5 by 1–2 μm) borne on adelophialides (reduced phialides lacking a basal septum); and *P. obovatum* colonies on potato flakes agar (at 25°C) demonstrate characteristic diffusible green pigment on reverse [20].

While in vitro culture enables more accurate morphological characterization of *Phialemonium* spp., the process is time-consuming and demands considerable technical expertise. With the recent development and application of nucleic acid amplification and sequencing techniques, the precise and speedy identification of *Phialemonium* spp. has become feasible. For example, PCR amplification of the internal transcribed spacer (ITS) regions followed by nucleotide sequencing contributed to the confirmation of the *P. curvatum* identity of case patient's isolates [22].

## 57.2 METHODS

### 57.2.1 SAMPLE PREPARATION

Clinical specimens suspected of dematiaceous fungi are treated with 10% KOH or stained with calcofluor white and observed microscopically for septate hyphal elements. Fungal isolation is performed on SDA with chloramphenicol or potato dextrose agar (PDA) at 25°C, 35°C. Suspected *Phialemonium* isolates are subcultured on PDA at 25°C. Preliminary identification is based on macroscopic and microscopic features of the colonies.

After growth for 1–7 days on PDA slants, approximately 1 cm<sup>2</sup> of mycelia is collected by scraping the slant with a sterile stick in 1 mL of sterile H<sub>2</sub>O. The material is transferred to a 2 mL screw-cap tube, and the tube is centrifuged for 1 min at 6000 × g. If the mycelia do not pellet, the material is contained with a pediatric blood serum filter. After removal of supernatant, the material is resuspended in 200 μL of IDI sample buffer and transferred to the lysis tube containing glass beads. Lysis tube is vortexed for 5 min, and the tube is placed in a boiling water bath for 15 min. Following centrifugation for 5 min at 16,000 × g, the supernatant is stored at –20°C until PCR.

Alternatively, about 1 cm<sup>2</sup> of fungal material is transferred to a 2 mL Eppendorf tube containing a 2:1 (wt/wt) mixture of silica gel and Celite (silica gel H, Merck 7736/ Kieselguhr Celite 545; Machery) and 300 μL of TES buffer [2 g Tris (hydroxymethyl)-aminomethane, 0.38 g Na-ethylenediaminetetraacetic acid (EDTA), and 2 g sodium dodecyl sulfate in 80 mL of ultrapure water (pH 8)]. The fungal material is ground with a micropestle for 1–2 min. The volume is adjusted by adding 200 μL of TES buffer. After vigorous shaking and the addition of 10 μL of a 10 mg/mL concentration of proteinase K to the tube, the mixture is incubated at 65°C for 10 min. With the addition of 140 μL of 5 M NaCl solution, the mixture is combined with 1/10 volume (~65 μL) of CTAB (cetyltrimethylammonium bromide) buffer 10%, followed by incubation for another 30 min at 65°C.

One volume (~700 μL) of chloroform-isoamyl alcohol (vol/vol = 24/L) is added and mixed by inversion. After incubation for 30 min at 0°C (on ice water) and centrifugation at 14,000 rpm at 4°C for 10 min, the top layer is transferred to a clean Eppendorf tube. The sample is added with 225 μL of 5 M NH<sub>4</sub>-acetate and incubated for at least 30 min (on ice water) and centrifuged. The supernatant is transferred to a clean sterile Eppendorf tube and mixed with a 0.55 volume (~510 μL) of ice-cold isopropanol. After centrifugation for 7 min at 14,000 rpm and 4°C (or room temperature), the supernatant is decanted. The pellet is washed with 70% ice-cold ethanol two times and dried by using a vacuum dryer. The powder is resuspended in 48.5 μL of Tris-EDTA buffer with 1.5 μL of 10 mg of RNase/mL, incubated at 37°C for 15–30 min, and stored at –20°C until use [23].

Furthermore, *Phialemonium* isolates are grown on liquid complete yeast medium, and DNA may be extracted using the FastDNA Kit (Bio 101).

### 57.2.2 DETECTION PROCEDURES

Proia et al. [11] utilized universal primers V9G (5'-TACGTCCCTGCCCTTTGTA-3') and LR5 (5'-ATCC TGAGGGAAACTTC-3') to amplify the ITS region of nuclear ribosomal DNA followed by sequencing, leading to the identification of *Phialemonium* isolates.

#### Procedure

1. PCR mixture (50 μL) is composed of 25 pmol of each primer, 200 μmol of each dNTP, 1 U of Taq polymerase (Super Taq; HT Biotechnology), 1× standard PCR buffer, and 1 μL of genomic DNA extract.
2. Amplification is carried out with an initial 94°C for 2 min; 35 cycles at 94°C for 35 s, 55°C for 50 s, and 72°C for 2 min; and a final 72°C for 6 min.
3. After checking a portion of the PCR products on agarose gel, the remaining PCR products are purified with the GFX PCR DNA and Gel Band Purification Kit (Amersham Pharmacia).
4. The PCR products are sequenced with the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and (5'-TCCTCCGCTTATTGATATGC-3') [White, et al. 1990] by means of the BigDye terminator cycle sequencing kit (Applied Biosystems). Sequences are analyzed on an ABI Prism 3700 instrument (Applied Biosystems). AQ2
5. The resulting sequences are aligned against published sequences using Clustal X, version 1.81. Phylogenetic relationships of the taxa are estimated from the aligned sequences by the maximum parsimony criterion, as implemented in PAUP, version 4.0b10. Heuristic searches are performed using parsimony informative, unordered, and equally weighted characters; branch robustness is tested by means of 1000 search replications, each on AQ3



bootstrapped data sets. Starting tree(s) are obtained via stepwise, random sequence addition repeated 100 times (repeated 10 times in bootstrap analyses). A maximum number of 9000 trees (1000 trees in bootstrap analyses) are allowed [9].

### 57.3 CONCLUSION

The genus *Phialemonium* is a dematiaceous fungus (with melanin in the cell walls and septa in the hyphae) that demonstrates intermediate morphological features between the hyaline or nonpigmented genus *Acremonium* and pigmented genus *Phialophora*. Because *Phialemonium* hyphal pigmentation is only detectable in tissue with melanin-specific histological stain such as the Fontana-Masson silver stain, it may be also confused with other phaeohyphomycotic agents including *Alternaria alternata*, *Bipolaris spicifera*, *Exophiala jeanselmei*, and *E. spinifera*, which produce hyaline hyphae.

Along with an increasing occurrence of immunosuppression in human populations, invasive infectious diseases due to emerging pathogens such as *Phialemonium* species are reported with alarming frequency. Indeed, both species within the genus *Phialemonium*, *P. obovatum*, and *P. curvatum*, have been shown to cause endocarditis, fungemia, osteomyelitis, keratitis, and endophthalmitis, often with fatal outcome. Considering that many fungal organisms demonstrate varied sensitivity to antifungal drugs, it is necessary to correctly identify them to species level. As laboratory characterization of *Phialemonium* species based on their macroscopic and microscopic features is lengthy and technically demanding, molecular techniques such as PCR and sequencing have become an indispensable tool for their identification and detection.

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### AUTHOR QUERIES

- [AQ1] Please check the word ‘pheoid’ in the sentence beginning “The genus *Phialemonium* is considered as a ...” for correctness.
- [AQ2] Please check the sentence starting “The PCR...” for completeness. Please provide the second primer in this sentence.
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