17 *Piedraia*

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CONTENTS

17.1 INTRODUCTION

Clinical presentations of fungal infections in humans generally fall under the following four categories: (i) superficial, (ii) cutaneous, (iii) subcutaneous, and (iv) systemic mycoses. Superficial and cutaneous fungal infections are often restricted to the stratum corneum and its adnexal structures, with little or no tissue involvement.

Black piedra (piedra, meaning "stone" in Spanish) is a superficial fungal infection of humans caused by a dematiaceous fungus called *Piedraia hortae*. This infection typically affects the frontal scalp hair and facial hair, producing darkly pigmented hair-shaft nodules as large as a few millimeters in diameter.

Black piedra differs from white piedra (trichosporosis) in that white piedra is caused by basidiomycetous arthroconidial yeast *Trichosporon* spp., producing lightly pigmented, white to light-brown, loosely attached hair-shaft nodules with a soft texture on the scalp hair, pubic hair, axillary hair, beards, moustaches, eyebrows, and eyelashes. In addition, black piedra is found mainly in hot and humid tropical climate, whereas white piedra is prevalent in temperate and semitropical climates [1,2].

17.1.1 Classification and Morphology

The genus *Piedraia* is a dematiaceous (dark-walled) mold belonging to the family *Piedraiaceae*, order Capnodiales, subclass Dothideomycetidae, class Dothideomycetes, subphylum Pezizomycotina, phylum Ascomycota, kingdom Fungi. Being the only member in the family *Piedraiaceae*, the genus *Piedraia* consists of two species: *Piedraia hortae* and *Piedraia quintanilhae* [2]. While *Piedraia hortae* is a keratinolytic fungus, causing black piedra in man, *Piedraia quintanilhae* has been isolated from chimpanzees in Central Africa, and its pathogenicity in humans is unknown.

Piedraia hortae colonies are slow growing, small, folded, velvety, dark brown to black in color; reverse is black. Colonies may produce a reddish brown diffusible pigment and remain glabrous or covered with short aerial hyphae. Hyphae are septate, darkly pigmented, with intercalary chlamydoconidium-like cells. Ascostromata are pseudoparenchymatous structures of subglobose to irregular shape and black in color, with each ascostromata usually containing a single ascus. Asci (with readily dissolvable walls) are ellipsoid, solitary, or in clusters and contain eight ascospores. Ascospores (30–45 μm × 5.5–10 μm) are hyaline to darkly pigmented, one celled (aseptate), fusoid, curved, and tapering toward both ends to form the typical whip-like appendages. *P. hortae* is one of only a few pathogenic human fungi that generate sexual spores in its parasitic phase [3,4]. *Piedraia quintanilhae* differs from *Piedraia hortae* morphologically in that its ascospores do not have appendages [5].

17.1.2 Clinical Features and Pathogenesis

Piedraia is a filamentous fungus commonly present in soil as well as stagnant water and crops in tropical regions of the world. *Piedraia hortae*, the etiologic agent of black piedra, has been isolated in tropical South and Central Americas, Southeast Asia, and Africa. The hot humid environment together with the habit of using plant oil on hair facilitates *Piedraia hortae* growth [6–8].

Superficial infection of hair shafts by *Piedraia hortae* results in the formation of asymptomatic, brown to black, stone-like concretions (nodules) of up to a few millimeters in diameter on the scalp (frontal, occipital, and parietal) and facial hair but not beard, moustache, and pubic hair (commonly known as black piedra). Very firmly attached to the hair shaft, the nodules are composed of ascostromata, which are the fruiting body of the fungus containing subglobose asci and aseptate ascospores in groups of

eight covered with a gelatinous sheath. The periphery of the nodule has regularly aligned hyphae and arthroconidia. The hard, darkly pigmented hair-shaft nodules of black piedra have a gritty feeling and may produce a metallic sound when hair is brushed. They rarely produce hair breakage in severe cases.

Coimbra et al. [9] conducted an epidemiological survey of black piedra among Zoró Indians in the Brazilian Amazon, who use plant oil on hair as a custom, and showed that 74 (56.9%) of the 130 individuals had the infection. Gip [10] documented a case of black piedra in a 23-year-old Swedish man after his return from 4 months' stay in India. The patient presented with typical clinical signs of black piedra of his scalp. Black nodules were found around the hair shafts, and the crushed nodules revealed numerous asci and ascospores on microscopy. *Piedraia hortae* was isolated from the concretions. After treatment with oral terbinafine 250 mg daily for 6 weeks, nodules disappeared.

Upon examination of the ultrastructural pattern of human hair infection by *Piedraia hortae* in vivo, Figueras et al. [11] demonstrated that the fungus has the capacity to destroy the cuticular layers of the hair and to penetrate deeply into the cortex. The main reasons that guarantee the long survival of the fungus, and therefore the chronic course of the disease, are possibly the slow rate of keratin degradation at the cortex together with the compacted stromatic organization of the nodules.

17.1.3 Diagnosis

Besides *Piedraia hortae*, a number of other fungi are capable of infecting human scalp and facial hair. Chief among them are white piedra (trichoporosis)-causing basidiomycetous arthroconidial yeast *Trichosporon* spp. (of which *Trichosporon ovoides*, *Trichosporon inkin*, *Trichosporon mucoides*, and *Trichosporon asahii* are linked to white piedra, with *T. inkin* likely on pubic hair and *T. ovoides* on head hair) and tinea capitis-causing dermatophytes *Trichophyton tonsurans*, *Microsporum audouinii*, and *Microsporum canis* [12]. Occasionally, *Acremonium* spp., *Brevibacterium* spp., and coryneform bacteria may also be involved in genital white piedra [13]. Other differential consideration is hair lice (pediculosis capitis).

Clinically, black piedra due to *Piedraia hortae* generates darkly pigmented, firmly attached hair-shaft nodules (concretions) on the scalp and facial hair, whereas white piedra due to *Trichosporon* spp. induces lightly pigmented, white to light-brown, loosely attached hair-shaft nodules (concretions) on the scalp hair as well as hair in other locations (including pubic hair, axillary hair, beards, moustaches, eyebrows, and eyelashes) [11,14]. The nodules of black piedra consist of ascostromata (fruiting body) with subglobose asci and aseptate ascospores in groups of eight inside the hyphae and arthroconidia in the periphery; the nodules of white piedra have darkly stained hyphae, blastoconidia (2–8 µm long), and arthroconidia fixed to the hair shaft [15,16]. Further, black piedra is distributed mainly in hot and humid tropical climate, whereas white piedra is endemic in temperate and semitropical climates [2,3,17].

On the other hand, tinea capitis due to dermatophyte fungi *Trichophyton tonsurans*, *Microsporum audouinii*, and *M. canis* does not form nodules on hair shaft. These organisms affect the base of the hair shaft and the follicle. *Trichophyton tonsurans* grows inside hair shaft (endothrix-growing), with arthroconidia forming within hair shaft; *Microsporum audouinii* and *Microsporum canis* grow outside hair shaft (ectothrix-growing), with numerous arthroconidia surrounding hair shaft and destroying its cuticle.

Direct microscopic examination of infected hair in 10% KOH allows a clear differential diagnosis of black and white piedra, as well as eggs of pediculosis. White and black piedra can also be distinguished from the color of the nodules (i.e., white to light brown, pale greenish or yellowish in white piedra and black in black piedra) and by the presence of ascospores and asci in black piedra and its absence in white piedra.

In culture, *Piedraia hortae* grows slowly at 25°C on Sabouraud's dextrose agar. As *Piedraia hortae* is uninhibited by cycloheximide, it will grow in dermatophyte test media (DTM) incorporating cycloheximide (e.g., Mycosel, DTM). *Trichosporon* spp. grow well on Sabouraud's dextrose agar at 28°C–30°C. However, they are inhibited by cycloheximide and will not grow in DTM containing cycloheximide. Because *P. hortae* rarely produces ascospores on Sabouraud agar, special techniques using transplantation and biotine may be utilized to stimulate its formation of ascospores.

Piedraia hortae and other hair invading fungal organisms can be identified in a much rapid and precise manner through polymerase chain reaction (PCR) and sequencing analysis of internal transcribed spacer (ITS) and rRNA gene regions [18,19].

Treatment options for black piedra include shaving or cutting the hair and oral terbinafine. The compact nature of black piedra nodules may compromise the effectiveness of antifungal therapy [10,20,21].

17.2 METHODS

17.2.1 Sample Preparation

Hairs with visible nodules are plucked, sectioned, and stained with toluidine blue. A 10%–15% KOH solution is used to stain hair-shaft nodules on a glass slide; a fungal stain (e.g., chlorazol black E stain or Parker blue-black ink) is added to delineate the hyphae. Nodule is crushed, taking care not to break the coverslip. Microscopic observation of septate brown hyphae, asci, and fusiform ascospores confirms the diagnosis. Culture of the organism is carried out on media with antibacterial agents, as well as media with both antibacterial agents and cycloheximide.

For DNA extraction, fungal isolates are cultured in 20 mL of RPMI 1640 medium with L-glutamine but without sodium bicarbonate (Sigma-Aldrich) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS)

(Sigma-Aldrich). After 3–14 days of growth at 30°C under agitation (100 rpm), mycelium is transferred into a tube and washed in 40 mL of sterile distilled water. Mycelium is then stored at −20°C until use. DNA extraction is carried out by a glass bead lysis method. A measured quantity of 100 mg of mycelium is homogenized for 1 min in a tube containing 1 mL of lysis buffer (2% Triton X-100, 1% sodium dodecyl sulfate, 10 mM Tris–HCl pH 8, 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid [EDTA] pH 8), three 0.5 cm diameter glass beads (Sigma), and approximately 500 mg of 425–600 µm glass beads (Sigma). The homogenized mycelia are then snap-frozen in liquid nitrogen, thawed, and refrozen once. DNA extraction is then done with the DNeasy plant kit (QIAGEN) [22].

17.2.2 Detection Procedures

Pounder et al. [19] described a real-time PCR with SYBR green DNA binding dye and amplicon melting temperature analysis for fungal detection also using pan-fungal primers ITS1 forward (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 reverse (5′-TCCTCCGCTTATTGATATGC-3′). The identity of the fungi is verified by subsequent sequencing analysis.

Procedure

1. PCR mixture is composed of 1× Lightcycler FastStart DNA Master Hybridization Probes mixture (Roche Applied Science) (containing deoxynucleoside triphosphates, FastStart *Taq* DNA polymerase, and 1 mM $MgCl_2$, additional $MgCl_2$ is added to a final concentration of 4.6 mM), 0.4 µM each of ITS1 forward and ITS4 reverse primers, 1× SYBR green (Molecular Probes), and 3 µL template DNA.
2. Thermal cycling parameters include 95°C for 10 min; 50 cycles of 95°C for 5 s, 60°C for 20 s, and 76°C for 30 s; and a final extension at 72°C for 2 min.
3. The quality of the amplicon is determined using the derivative of the melt analysis curve (55°C–99°C, 45 s hold at 55°C, 5 s/°C) using the RotorGene 3000 (Corbett Robotics, Inc).
4. The amplified product is purified for bidirectional sequencing using ExoSAP-IT (USB Corp). Five microliters of Big Dye Terminator Ready Reaction Mix v. 1.1 (Applied Biosystems) is added to 4 µL of each primer (0.8 pmol/µL) and 3 µL of purified PCR product. Cycle sequencing is performed with a 9700 thermal cycler (ABI), using 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Sequencing reaction products are passed through a Sephadex G-50 fine column to remove unincorporated dye terminators. Purified sequencing reaction products are run on an ABI Prism 3100 Genetic Analyzer with a 50 cm capillary array.
5. Sequences are analyzed with the SmartGene Integrated Database Network software version 3.2.3 vr. SmartGene is a web-based software and database system with reference sequences derived from the National Center for Biological Information (NCBI) GenBank repository.

Note. In case that real time PCR instrument is not available, standard PCR may be performed with primers ITSI and ITS4, and the resulting amplicon is sequenced with the same primers. Sequence-based identifications are defined by percent identity: species, ≥ 99%; genus, 93%–99%; and inconclusive, ≤ 93%.

For strains producing discrepant identification between the methods based on phenotypic characteristics and ITS sequence analysis, the D1–D2 region of the large-subunit RNA gene is amplified with primers NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) and sequenced for species clarification.

17.3 CONCLUSION

Piedraia hortae is a keratinophilic dematiaceous fungus causing black piedra, which is characterized by the formation of black, firmly adhered nodules on scalp and facial hair. Crushed nodules mounted with KOH (10%–20%) display tightly packed, thick-walled fungal cells and asci containing two to eight single-celled, fusiform, slightly curved ascospores with a single polar filament at each end. Black piedra needs to be differentiated from white piedra (trichosporosis) caused by basidiomycetous arthroconidial yeast *Trichosporon* spp. as well as tinea capitis caused by dermatophyte fungi *Trichophyton tonsurans*, *Microsporum audouinii*, and *Microsporum canis*. White piedra is characterized by the formation of white to light brown, easily detachable nodules surrounding hair shaft. Apart from causing white piedra, *Trichosporon* spp. also have the capacity to induce systemic infections (termed trichosporonosis) in immunocompromised patients. Use of molecular techniques such as PCR and sequencing analysis enables accurate and rapid identification of *Piedraia hortae* and other hair invading fungal organisms.

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