Molecular Diagnosis of Subcutaneous *Pythium insidiosum* infection using PCR

Screening and DNA Sequencing

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ABSTRACT

*Pythium insidiosum* is an emerging human pathogen classified among brown algae and diatoms that can cause significant morbidity and mortality in otherwise healthy individuals. Here we describe a pediatric patient with pythiosis acquired in the southern United States, diagnosed by molecular screening and DNA sequencing of internal transcribed spacer region 1.
CASE REPORT

An otherwise healthy 14-year-old girl living in urban North Texas presented to an urgent care facility with a 2-week history of a progressively enlarging, erythematous bump on the medial aspect of her left lower leg. She denied recent travel, but was noted to have gone swimming in a swimming pool with signs of algal overgrowth. She was treated with incision and drainage and given a course of oral cephalexin. Although initially responding to oral antibiotics, over 2 weeks she again developed worsening local edema and erythema. The patient was switched to oral clindamycin but did not show improvement, prompting hospital admission about 3 weeks into her illness. She was administered intravenous clindamycin and an ultrasound of the area revealed soft tissue swelling with some patchy areas suggestive of fluid accumulation, but without clear signs of an abscess. Incision and drainage was performed and a drain was placed. Wound cultures were sent, and returned positive for *Staphylococcus intermedius* and *Pseudomonas aeruginosa*. Based on the culture sensitivities of those organisms antibiotic therapy was tailored to cefepime, tobramycin, and clindamycin. The patient showed clinical improvement and was discharged on oral ciprofloxacin 1 week after admission.

After approximately 5 days, erythema and edema around the wound had visibly increased and the patient was readmitted approximately one month after onset of her initial symptoms. She was administered intravenous meropenem and amikacin and incision and drainage was repeated, revealing an abscess measuring 10 cm by 15 cm. A drain was again placed, and wound cultures were sent for growth of bacteria, fungi, and acid-fast-bacilli. Histologic sections from debridement material demonstrated an acute
and chronic inflammatory infiltrate, interrupted by eosinophilic pools comprised of poorly formed granulomas with central necrosis admixed with neutrophils, eosinophils, and mineralized debris (Figure 1A). Rare hyphal elements on Gomori methenamine silver stain were identified (Figure 1B and 1C). Consequently, amikacin was discontinued, and the patient was started on liposomal Amphotericin B (250 mg IV qDay), with the addition of posaconazole (400 mg PO BID) after 4 days. Nevertheless, the patient continued to have worsening erythema, edema, and patchy fluctuance at the wound site. Cultures of debridement material remained negative for fungal growth following one week of incubation, and one formalin fixed paraffin embedded (FFPE) tissue block was therefore submitted for molecular characterization to the University of Washington Molecular Diagnosis Microbiology Section. During this time the patient underwent another incision and drainage, and the abscess was found to have enlarged to approximately 12 cm by 15 cm. Intravenous antibiotics were continued and a wound vacuum device was placed with thrice-weekly changes, yet the lesion continued to slowly enlarge and to show progressive inflammation and tissue necrosis. Her worsening condition prompted more extensive surgical debridement one week after the previous procedure. Histology revealed necrotizing and suppurative granulomas with more abundant hyphal elements. Magnetic resonance imaging (MRI) demonstrated extensive inflammation along the subcutaneous tissue and muscle layers up to the level of the mid-thigh, with occlusion of the posterior tibial artery.

DNA extraction from FFPE material was performed as reported previously (7). All PCR products were separated by electrophoresis using a 1% agarose gel and stained for visualization. PCR using primers directed against ITS region 1 (primers ITS1 and ITS2)
yielded a product (Figure 2A) which was subjected to bidirectional sequencing and contig assembly as described elsewhere (12). Results were submitted to a GenBank Basic Local Alignment Search Tool (BLAST) search. The top 25 matches from the BLAST search of this sequence (GenBank accession JQ305801) corresponded to various *P. insidiosum* isolates, with the highest-ranked match identical to the query sequence (GenBank accession 284073108: 100% identity, 100% query coverage, BLAST E value 1.78e-141, alignment length 276 bp). Confirmatory sequencing was repeated on the FFPE tissue block and also performed on two additional FFPE blocks, using primers flanking both ITS1 and COXI (cytochrome oxidase c subunit 1, primers OomCoxI-Levup and OomCoxI-Levlo (16)) regions. In all three blocks amplification and sequencing of ITS1 resulted in contigs identical to that obtained from the initial block. COXI amplification was successful from only one of the three blocks (Figure 2B), and a BLAST search of the resultant sequence (GenBank accession JQ305799) revealed significant homology to *P. insidiosum* (top 3 matches, representative GenBank accession HQ708612.1: 99% identity, 97% query coverage, BLAST E value 0.0, alignment length 679 bp).

Following molecular characterization, a pure culture of the infectious organism was subsequently obtained by placing unprocessed debridement material directly onto Sabourad's agar plates, and incubating at 30°C (Figure 1E and 1F). We conclude that standard processing of infected tissue (by mincing) is physically disruptive to the organism. DNA was extracted from samples of this culture using Ultraclean Microbial DNA Isolation Kit (Mo-Bio), and submitted for molecular characterization using primers to amplify full-length ITS (ITS1 and ITS2 sequences) (14) (Figure 2C), COXI (Figure 2B), and 28s rRNA sequences (14) (Figure 2D). Sequences of both ITS1 and COXI
identically matched those generated from tissue blocks. The top match for the 28s sequence data (GenBank accession JQ305800) was the corresponding sequence from \textit{P. insidiosum} isolate CBS 574.85 (GenBank accession HQ665273.1: 99% identity, 99% query coverage, BLAST E value 0.0, alignment length 729 bp), and the closest 22 matches for full-length ITS sequence (GenBank accession JQ305801) were each against \textit{P. insidiosum} isolates (top match, GenBank accession AY151165.1: 99% identity, 100% query coverage, BLAST E value 0.0, alignment length 797 bp).

Following initial molecular identification approximately 3.5 weeks after admission, a diagnosis of presumptive \textit{P. insidiosum} soft tissue infection was made and antimicrobial treatment was optimized against that agent (18, 19). Therapy was converted to micafungin (150 mg IV qDay), terbinafine (500 mg PO qDay), minocycline (100 mg PO BID), and posaconazole (200 mg PO QID). The patient underwent extensive circumferential debridement, revealing invasion of the tibial artery and vein. Limb salvage was next attempted by local debridement followed by potassium iodide soaked gauze dressings for one week. Additionally, an investigational therapeutic \textit{P. insidiosum} immunotherapy (23) was initiated 4 times weekly. Despite these interventions, extensive muscular necrosis with overlying eschars and worsening soft tissue edema developed, prompting further debridement 5, 6, and 6.5 weeks after admission. Pathology specimens from those procedures demonstrated ongoing inflammation with poorly-organized granulomas, eosinophils, worsening necrosis, arterial thrombosis, and more abundant fungal septate and nonseptate hyphae. The highest concentration of organisms was observed colonizing the surface of gangrenous tissue (Figure 1D).
Given the extensive involvement of the infection, and the generally poor prognosis of advanced pythiosis, the decision was made to perform an above-the-knee-amputation. Histological sections from the procedure showed deep extension of the infection into skeletal muscle, although the margins and popliteal lymph nodes were found to be clear of the organism. Following amputation the patient showed immediate clinical improvement and her wound healed appropriately. The patient was discharged after 10 days of observation without antimicrobial therapy, and remained free from signs of residual infection on follow-up.

*Pythium insidiosum* is a protist that inhabits bodies of fresh water in tropical, subtropical, and temperate climates (6, 11). It is a member of the oomycete (perosporomycetes) class, unusual organisms that share several morphologic similarities to fungi: many are filamentous, sporulating organisms, marked by thick cell walls. Regardless, oomycetes are ecologically, genetically, and physiologically distinct from fungi. Phylogenetic analyses based on a variety of genetic loci have established that the lineage falls among the stramenopiles, which includes brown algae and diatoms, representing an evolutionarily isolated branch of the eukaryotes (16, 17, 21). In biochemical support of this classification, oomycete cell walls are composed mostly of cellulose and polymerized beta-1,3-glucan, common for algae but dissimilar to the chitin (beta-1,4-N-acetylglucosamine) characteristic of true fungi (9). A number of oomycete species are known agents of disease in plants or in animals (including insects and fish), yet *P. insidiosum* and recently recognized *P. aphanidermatum* (4), are the only members
of the genus capable of infecting mammalian hosts (4, 13, 16).

Long recognized as a veterinary pathogen primarily affecting horses and canines (6), the first human cases of *P. insidiosum* infection were reported in 1985 (5, 10). Pythiosis (or “swamp cancer”) is now regarded as an emerging human disease. Less than 150 cases of human pythiosis have been reported to date, with the great majority occurring in Thailand, where the disease is considered endemic (10). Regardless, *P. insidiosum* has a wide geographic distribution, and there exist case studies of human pythiosis contracted from environmental sources across the globe, including rare cases in the United States (6, 18) (15). In this case report the patient presumably acquired pythiosis from an artificial water reservoir in an urban setting (a neglected swimming pool with algal overgrowth).

If not recognized and treated early, pythiosis can rapidly progress to a life-threatening infection in otherwise healthy individuals (15) (6). The diagnosis was further complicated in this case by the initial presence of bacterial superinfection. In immunocompetent persons, infection with *P. insidiosum* typically affects vascular, ophthalmic, or subcutaneous tissues, but it may present as systemic disease in thalassemic or leukemic patients (22). Pythiosis can manifest with a variety of different symptoms depending on the site and severity of infection (6, 10). Vascular disease typically favors the arteries, and may advance to cause thrombosis or aneurysm. Infection of the eye or subcutaneous tissues produces progressive granulomatous and ulcerating lesions. Given its unusual biology the organism is resistant to antifungal and antimicrobial agents: radical surgery is a mainstay of treatment for pythiosis. Although particular drug
cocktails or an experimental immunotherapy have been suggested to be of some benefit (18-20, 23), in our patient medical management, including therapeutic immunotherapy (23), was unsuccessful.

There currently exists no “gold standard” method for the clinical diagnosis of *P. insidiosum* infection. Even isolating the organism in pure culture does not guarantee an accurate identification: the procedure is both time consuming and technically challenging, and the organism may fail to generate characteristic zoospores when cultured *in vitro* (3, 6). Serological methods have been described, including complement fixation, immunodiffusion, fluorescent antibody tests, immunoblots, enzyme-linked immunosorbent assays (ELISA), and hemagglutinin tests (8). However, these techniques display variable sensitivity and specificity, especially during early infection when the immune response is not fully mounted (8). Definitive diagnosis can be achieved using molecular methods, including species-specific nested PCR (3), or DNA sequencing of variable regions (1, 2).

In general, sequence analysis of internal transcribed spacer (ITS) regions has proven to be a powerful tool for molecular diagnosis of fungal and parasitic pathogens (7, 12, 14). Situated between highly conserved rRNA structural genes, ITS sequences are present in all eukaryotes. These domains can be targeted for species-independent PCR amplification using “universal” primers directed against conserved sites in flanking rRNA sequences. Because they do not encode functional rRNAs or proteins, ITS sequences have diverged considerably among organisms: in many cases they harbor enough variability to allow discrimination between even closely related species (14). ITS1 (between the 18S and 5.8S rRNA genes), in particular, has been extensively
validated for use in molecular identification. Given the morphological similarities between oomycetes and fungi, it is fortuitous that these primer binding sites remain highly conserved in *P. insidiosum*: as in this case, and as previously recognized (1, 2), standard molecular workup of a presumptive fungal pathogen can identify *P. insidiosum*. Here, we have demonstrated that ITS1 sequencing is robust enough to provide a definitive diagnosis even from small amounts of material that has been formalin fixed and paraffin embedded.

We additionally sequenced a separate variable locus, COXI, which previously proved useful in identifying *Taenia solium* infection (7). Studies examining COXI across oomycete species has suggested that sequencing ITS with COXI is synergistic, and in some cases COXI provides greater discriminative power among species than ITS alone (16). The suitability of COXI for clinical identification of oomycete infection has previously been proposed (16), yet this is the first instance where it has been implemented in clinical practice. Nevertheless, probably due to the large size of the COXI amplicon, we found that it was more difficult to generate COXI PCR products from FFPE tissues than for ITS1. We were able to more sensitively detect the presence of *P. insidiosum* in a larger number of tissue blocks (all three) using universal ITS1 primer sequences, whereas COXI amplified from only one block.

Because *P. insidiosum* is an emerging pathogen it is anticipated that the frequency of infections with this agent will increase in the future. This case report illustrates that the diagnosis of pythiosis should be considered in any patient with suggestive exposure history and presumptive fungal forms by microscopy, especially if the infection is refractory to standard antifungals. The development of diagnostic tests for *P. insidiosum*...
remains an unsolved challenge; however, molecular identification can be performed within a clinically meaningful timeframe and allows for definitive diagnosis. We have found ITS1 to be a particularly robust target for molecular identification. More broadly, this report illustrates the value of molecular diagnosis in the context of an infectious agent that is unknown, difficult or impossible to culture, unexpectedly recalcitrant to treatment, or as in this case study, exhibits all three properties simultaneously. PCR screening and DNA sequencing of variable regions, utilizing primer binding sites directed against highly conserved loci, provides a strategy to allow definitive identification of a range of pathogens, suspected or unsuspected (12), efficiently providing information that can inform patient care. A molecular screening approach is particularly helpful in instances where there is an extensive differential diagnosis or where pathogen-specific testing is suboptimal or unavailable (12).

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REFERENCES.


FIGURE LEGENDS:

**Figure 1. Histological sections and pure culture of *P. insidiosum***. A. Section of early debridement material using hematoxylin and eosin staining. Arrowheads indicate poorly formed granulomatous response. Original magnification 4×. B. Histology of early debridement material, arrowheads indicating rare hyphal elements on Gomori methenamine silver stain. Original magnification 10×. C. Same preparation as in B., 40× original magnification. D. Histologic section demonstrating abundant organisms colonizing the surface of gangrenous tissue, visualized as in B. E. Sandwich preparation of cultured organism stained with lactophenol cotton blue, original magnification 100×. Sparsely septate hyphae are observed, with no fruiting bodies. F. Same preparation as E., original magnification 400×.

**Figure 2. Molecular characterization of *P. insidiosum***. All panels were imaged on a 1% agarose gel alongside a 1kb plus ladder (Invitrogen). Represented are PCR products from amplification of: A. ITS1 (tissue block in duplicate), B. COXI (all samples in duplicate), C. full-length ITS, and D. 28s rRNA. T, FFPE tissue block; C, cultured organism; I, inhibition control (equal mixture sample and positive amplification control); (+), positive amplification control (*Candida sorbosivorans* DNA); (-), negative control (no template).