Molecular Assays for Detecting Aphanomyces invadans in Ulcerative Mycotic Fish Lesions

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The pathogenic oomycete Aphanomyces invadans is the primary etiological agent in ulcerative mycosis, an ulcerative skin disease caused by a fungus-like agent of wild and cultured fish. We developed sensitive PCR and fluorescent peptide nucleic acid in situ hybridization (FISH) assays to detect A. invadans. Laboratory-challenged killfish (Fundulus heteroclitus) were first tested to optimize and validate the assays. Skin ulcers of Atlantic menhaden (Brevoortia tyrannus) from populations found in the Pamlico and Neuse River estuaries in North Carolina were then surveyed. Results from both assays indicated that all of the lesioned menhaden (n = 50) collected in September 2004 were positive for A. invadans. Neither the FISH assay nor the PCR assay cross-reacted with other closely related oomycetes. These results provided strong evidence that A. invadans is the primary oomycete pathogen in ulcerative mycosis and demonstrated the utility of the assays. The FISH assay is the first molecular assay to provide unambiguous visual confirmation that hyphae in the ulcerated lesions were exclusively A. invadans.

Since the early 1980s, estuarine fish, primarily Atlantic menhaden (Brevoortia tyrannus), along the east coast of the United States have experienced seasonal epidemics of deep, aggressive skin ulcers known as ulcerative mycosis (UM) (13, 33). UM has been reported regularly in the spring and fall from the St. Johns River in Florida, the Neuse and Pamlico Rivers in North Carolina, and some tributaries of the Chesapeake Bay (1, 4, 17, 35). Pathological surveys of skin ulcers revealed that most contained oomycetes (filamentous protists that physically resemble fungi) (2, 24, 34). Specialized culture techniques were developed to isolate and identify these oomycetes (3, 13, 46). A number of species belonging to genera as diverse as Aphanomyces, Saprolegnia, and Achlya were recovered (4, 12, 40). When tested, only one species, Aphanomyces invadans (synonymous with Aphanomyces piscicida), was pathogenic (3, 22, 23). This species occurs worldwide and infects both wild and cultured fish, often leading to mass fish mortality (4, 9, 27, 40). It was originally documented as the cause of mycotic granulomatosis of ayu (Plecoglossus altivelis altivelis) in Japan (18) and more recently as the cause of epizootic ulcerative syndrome or red spot of striped snakehead (Channa striata) and striped mullet (Mugil cephalus) from southern Asia and Australia (9, 15, 21, 30, 40). These skin diseases (mycotic granulomatosis, epizootic ulcerative syndrome, red spot, and UM) are clinically identical and occur in either freshwater or estuarine fish.

Routine identification of oomycetes directly from UM lesions is difficult because the species present in the lesions do not form the specialized reproductive structures required to differentiate one species from another. Thus, oomycetes must be cultured from the lesions and the reproductive structures must be formed before species identifications can be made. The culture recovery rate of oomycete pathogens typically is very low, often ≤10% (12, 13). Other microbes that are commonly present at high levels in UM lesions, especially bacteria, outcompete slow-growing species such as A. invadans in culture (40). Even if successfully cultured, oomycetes often fail to produce the sexual structures needed for identification to species. These cultural and identification problems have restricted our understanding of the biology, pathogenesis, and epidemiology of this emerging aquatic disease and have limited the identification of this pathogen to a relatively small number of fish with UM. At present, only two A. invadans isolates have been reported in culture from the United States (3, 12).

Our objectives in this study were to develop species-specific PCR and in situ hybridization assays to rapidly detect and identify A. invadans and to use the assays to screen populations of infected fish. In recent years, other PCR assays have been developed to detect A. invadans from formalin-preserved fish samples and from artificially infected fish (3, 38). These assays, however, have not been used for large-scale screening of fish populations. They also lack the visual capacity of fluorescent peptide nucleic acid in situ hybridization (FISH), which can be...
used to distinguish *A. invadans* from other oomycete species that may be present in UM lesions.

**Materials and Methods**

Collection and storage of ulcerated fish. Atlantic menhaden were collected (September 2004) by cast net from the Pamlico (35°28'41.9988"N, 76°49'47.3801"W) and Neuse (34°58'15.7801"N, 76°55'25.6239"E) Rivers, North Carolina. Fifty arbitrarily selected fish, exhibiting typical UM skin ulcers (34), were placed in plastic bags and transported to the laboratory on ice. Control fish without lesions were also collected. Specimens that were not necropsied and assayed immediately were frozen whole at −80°C.

Oomycete isolates and sporulation. Oomycete isolates used to validate the molecular assays (Table 1) were either obtained from the American Type Culture Collection (ATCC) or cultured from lesioned fish tissue by standard methods (4, 46). Isolates from Florida were shipped as needed on agar slants to the National Oceanographic and Atmospheric Administration Center for Coastal Fisheries and Habitat Research Laboratory, Beaufort, NC. All isolates were maintained at 24°C on Griffin's glucose-yeast (GY) broth or Griffin's GY agar (9, 10). Isolates were subcultured on GY agar slants at 24°C monthly, and spore suspensions were stored at 4°C until needed.

Experimental infections. Killifish (*Fundulus heteroclitus*) were infected artificially with *A. invadans* to provide material for validating and optimizing the PCR and FISH assays. Thirty healthy adult fish obtained from a Sparta marina adjacent to the Center for Coastal Fisheries and Habitat Research Laboratory were divided into three treatment groups (10 fish per group). The fish were acclimated to 21°C and a salinity of 4 practical salinity units for 1 week in separate 80 liter aquaria. Fish were fed flake food daily. Water quality was monitored and recorded daily for 3 days in sterile 50-ml plastic, conical, centrifuge tubes at 24°C. Cultures were checked weekly for growth of the fish. At the end of this period, the fish were euthanized with >500 mg · liter⁻¹ of MS 222; lesioned tissues (skin and muscle) were removed aseptically by standard necropsy procedures. Tissues that were not used immediately were stored at −80°C until needed.

DNA extraction, PCR, and sequencing protocol. The ClustalX algorithm was used to align rDNA gene sequences, available from GenBank, of *Aphanomyces sp.* (accession numbers AF396683, AJ238655, M32705, J202951, AJ238662, and AJ238656). Oomycete primers (OMYC 18SF and OMYCR) were designed for the amplification of the entire small subunit (SSU), the first internal transcribed spacer region (ITS1), the 5.8S rRNA, and the D1/D2 domains of the large subunit (LSU) rRNA gene. DNA was extracted from an *A. invadans* culture originally isolated from an Atlantic menhaden from the Great Wicomico River, Virginia. Actively growing colonies in GY broth were transferred to sterile 100-mm petri dishes, and the GY broth was decanted from the dish. Hyphal tips were excised with a sterile scalpel blade (total of ~250 mg) and transferred to a microcentrifuge tube for DNA extraction with an UltraClean soil DNA extraction kit (Mo Bio Laboratories, Inc., Solana Beach, Calif.), following the manufacturer’s protocol. The amplification reaction mixture contained 100 mM Tris-HCl (pH 8.3), 15 mM MgCl₂, 750 mM KCl, 25 pM each primer, 2.5 mM each deoxynucleoside triphosphate, 0.5 U of Platinum Taq DNA polymerase (Invitrogen Life Technologies, Rockville, Md.), and 20 ng of genomic DNA in a total reaction volume of 50 μl. The DNA was amplified in a Robocycler (Stratagene, La Jolla, Calif.) under the following cycling conditions: 2 min at 95°C, 35 cycles, each consisting of 30 s at 95°C, 45 s at 56°C, 2.5 min at 72°C, and a final extension of 5 min at 72°C. A 0.5-μl aliquot of each PCR was evaluated by agarose gel electrophoresis. PCRs containing specific products were purified with the QIAquick PCR purification kit (QIAGEN, Valencia, Calif.), quantified spectrophotometrically, and sequenced with an ABI377 DNA sequencer (Applied Biosystems, Foster City, Calif.). The resulting DNA sequences for *A. invadans* were deposited in the GenBank nucleotide sequence database (Accession No. AF396683).

**Development of FISH assays.** We used peptide nucleic acid (PNA) probes, which are neutrally charged synthetic molecules that mimic the structure of both DNA and RNA.
DNA and RNA (5, 31, 47) but have a higher melting temperature per base pair than do conventional RNA or DNA probes. The A. invadans SSU rRNA gene sequence obtained in this study was aligned with the SSU rRNA gene GenBank sequences listed above to design the species-specific probe. A unique hybridization site was identified (bp 621 to 635; AF396684), and a species-specific PNA probe for A. invadans with a 5’ fluorescent (FLU) conjugate was manufactured (5’-FLU-GTACACATCTTGCG-3’, designated Ainv-FLU3; Applied Biosystems, Bedford, Mass.).

In situ hybridization procedures were adapted from those developed for dinoflagellates (28, 29). Cultured hyphae, cultured zoospores, and infected tissues were fixed to preserve the integrity of the cells and to prevent RNA degradation. For cultured hyphae, the GY broth was decanted from culture tubes and the hyphae were rinsed twice for 10 min each time with 20 ml of sterile, deionized water in the culture tubes. After the second rinse, 25 ml of an ethanol-saline fixative was added to the tube to preserve the hyphae. The ethanol-saline fixative contained 44 ml of 95% ethanol, 10 ml of deionized H₂O, and 6 ml of 25X SET buffer (3.75 M NaCl, 25 mM EDTA, 0.5 M Tris HCl [pH 7.8]). Cultured hyphae were fixed for at least 30 min prior to hybridization.

Fixation of zoospore cultures was similar, except that the zoospores were allowed to settle for 15 min prior to fixation. The culture medium was removed with a pipette, leaving 5 ml of a concentrated zoospore suspension. Thirty milliliters of ethanol saline was used to fix the zoospores for at least 30 min before hybridization was initiated.

Fixation and permeabilization of A. invadans-infected fish tissue were optimized by using experimentally infected F. heteroclitus and lesioned material from naturally infected Atlantic menhaden. To minimize RNA degradation, fixation and hybridization procedures were carried out as soon as possible after the fish were collected. The hyphae were also removed from naturally infected fish and assayed to check for cross-reactivity and to serve as negative controls. Tissue (~20 mg) was dissected from the periphery of the lesions with sterile scalpel blades and placed in individual wells of a 24-well microtiter dish. One milliliter of ethanol-saline fixative containing 3% polyoxyethylene sorbitan monolaurate (Tween 20) was added to each well of a 24-well microtiter plate. Cultured hyphae were placed in 0.5 ml of hybridization buffer containing 3% Tween 20. The hybridization solution was removed, and the samples were washed (twice for 15 min each time) with 0.5-ml aliquots of hybridization buffer (5X SET buffer, 0.1% (vol/vol) Igepal CA-630 (Sigma, St. Louis, Mo.), and 25 μg · ml⁻¹ poly(A) (Sigma)). The hybridization buffer was washed, and the hyphae were resuspended in 0.5 ml of hybridization buffer containing 20 nM of the Ainv-FLU3 probe. The probe sequences listed above to design the species-specific probe. A unique hybridization site was identified (bp 621 to 635; AF396684), and a species-specific reverse primer site located in the ITS1 region. PCR amplification using the A. invadans primers Ainv-2F (5'-CTCAGTGAGTAAACCGGTG-3') and Ainvad-ITSR1 (5'-GGCTAAGGTTGATGATGATG-3') generated a 234-bp product.

PCR assays of ulcerated tissues. The primer pairs were tested for cross-reactivity to DNA extracted from other oomycetes (Table 1) and from nonlesioned fish (B. tyrannus and F. heteroclitus). DNA was also extracted from ulcerated tissues (10 mg) removed from 50 Atlantic menhaden with a DNeasy Tissue kit (QIAGEN) following the manufacturer’s protocol (Qiagen). DNA (50 ng) from each lesioned sample was added to the PCR mixtures and amplified as described above. Each PCR assay included a positive control, a negative control, a blank DNA extraction control, and a PCR inhibition control. The positive control incorporated 30 ng of A. invadans genomic DNA into an arbitrarily chosen ulcerated menhaden DNA sample. The inhibition control ensured that negative PCR were not due to PCR inhibition but to the absence of A. invadans DNA.

Nucleotide sequence accession number. The SSU-to-5' LSU rRNA gene sequence for A. invadans was submitted to GenBank and assigned accession number AF396684.

RESULTS

The A. invadans FISH probe was first tested for species specificity and cross-reactivity by hybridization to three strains of A. invadans and to nine related oomycetes (Aphanomyces spp., Saprobngenia spp., and Achlya spp.) sequenced available from GenBank (accession numbers AF396683, AF396684, AF396685, AF218159, and AF310185). The oomycete ITS regions contained a large number of unique species-specific primer sites relative to the flanking SSU, 5.8S, and LSU genes. The PCR assay utilized a species-specific forward primer site located near the 3’ end of the SSU gene and a species-specific reverse primer site located near the 5’ end of the LSU gene for amplification using the A. invadans primers Ainv-2F (5’-CTCAGTGAGTAAACCGGTG-3’) and Ainvad-ITSR1 (5’-GGCTAAGGTTGATGATGATG-3’) generated a 234-bp product.

PCR mixtures to confirm that the reagents were not contaminated. The blank extractions were performed with light and epifluorescence microscopy. Camera and microscope settings for epifluorescence analysis were held constant so that comparability analyses of relative fluorescence intensity could be made between probed and nonprobed specimens.

Each FISH assay included a positive, a negative, and a “no-probe” control. The positive control consisted of a PNA probe (Eu11, 5'-FLU-ACCAGACCT GCCCTCC; bp 509 to 524; AF396684) and a species-specific probe for A. invadans with a 5’ fluorescent (FLU) conjugate was manufactured (5’-FLU-GTACACATCTTGCG-3’, designated Ainv-FLU3; Applied Biosystems, Bedford, Mass.).

Microscopy and image acquisition. High-resolution photomicroscopy of the in situ hybridization experiments was conducted with an Orca II digital camera (Hamamatsu Photonics, Hamamatsu, Japan) and a Sony (Tokyo, Japan) 3CCD color video camera. The cameras were mounted on a Nikon (Tokyo, Japan) TE300 Eclipse inverted microscope. The cameras were interfaced with a desktop computer driven by Metamorph image acquisition and analysis software from Universal Imaging (Downington, Pa.). Differential interference contrast and epifluorescence microscopy (fluorescein isothiocyanate excitation (λem) and emission (λex) settings were as follows: λex = 460 to 500 nm; λem = 510 nm; dichroic long-pass beam splitter = 505 nm) were used to visualize the specimens.

In the first time a sample was thawed. In contrast, several cycles of freezing and thawing did not affect the sensitivity of the PCR assays.

PCR controls contained 25 pM each primer, 2.5 mM each deoxynucleoside triphosphate, 0.5 U of Platinum Taq DNA polymerase (Invitrogen), and 20 ng of genomic DNA for a total volume of 50 μl. Amplifications were carried out in a Robocycler with the following program: 2 min at 95°C, 35 cycles, each consisting of 20 s at 95°C, 30 s at 54°C, and 45 s at 72°C, and a final extension of 5 min at 72°C. An aliquot (5 μl) from each amplification was analyzed by agarose gel electrophoresis. The size of the PCR product was estimated by comparisons with a 100- or 123-bp molecular weight ladder (Promega, Madison, Wis., and Roche, Basel, Switzerland, respectively).

PCR assay of ulcerated tissues. The primer pairs tested for cross-reactivity to DNA extracted from other oomycetes (Table 1) and from nonlesioned fish (B. tyrannus and F. heteroclitus). DNA was also extracted from ulcerated tissues (10 mg) removed from 50 Atlantic menhaden with a DNeasy Tissue kit following the manufacturer’s protocol (Qiagen). DNA (50 ng) from each lesioned sample was added to the PCR mixtures and amplified as described above. Each PCR assay included a positive control, a negative control, a blank DNA extraction control, and a PCR inhibition control. The positive control incorporated 30 ng of A. invadans genomic DNA into an arbitrarily chosen ulcerated menhaden DNA sample. The inhibition control ensured that negative PCR were not due to PCR inhibition but to the absence of A. invadans DNA.

Nucleotide sequence accession number. The SSU-to-5' LSU rRNA gene sequence for A. invadans was submitted to GenBank and assigned accession number AF396684.
ded in the infected tissues exhibited the same aseptate branching pattern as was observed for *A. invadans* grown in culture. Killifish receiving the sham injections or injected with the nonpathogenic *Aphanomyces* sp. 84-1240 had a 100% survival rate and did not show any signs of infection or lesion formation. Samples of *A. invadans*-infected killifish tissue were used to empirically determine optimal hybridization conditions for the FISH probes.

Validation of *A. invadans* FISH assay. Fifty Atlantic menhaden exhibiting typical UM ulcers were assayed with the FISH probes. A universal eukaryote probe was used as the positive control, ensuring that the hyphae were permeabilized and that the rRNA was intact (Fig. 3A). The *A. invadans*-specific probe successfully hybridized to hyphae that typically spread throughout the tissue samples (Fig. 3B). The negative control probe was specific for the dinoflagellate *Pfiesteria piscicida* and was used to assess nonspecific probe hybridization, which did not occur in any of the experiments (Fig. 3C). A “no-probe” control assessed autofluorescence of the menhaden tissue and of the hyphae (Fig. 3D). All of the UM menhaden were positive for *A. invadans* by the FISH assay. Tissues assayed from nonlesioned fish were negative.

Validation of *A. invadans* PCR assay. Experiments testing the potential cross-reactivity of the *A. invadans*-specific PCR assay were performed with genomic DNA of other oomycete species (Table 1). These experiments confirmed that the *A. invadans* PCR assay was species specific and did not amplify DNA from related oomycete species (Fig. 4A). DNA was ex-
Aphanomyces invadans was detected by PCR in every lesioned tissue sample, exactly matching the FISH assay results (Fig. 4B). Tissues from nonlesioned fish were negative by the PCR assay.

**DISCUSSION**

Ulcerative mycosis is a serious fish disease caused by the pathogenic oomycete (water mold) *A. invadans*. This study describes the development of FISH and PCR assays that can rapidly detect *A. invadans* from naturally infected fish tissues. The specificity of the FISH assay was tested using a panel of related oomycetes, including four other *Aphanomyces* taxa (Fig. 1). After specificity was confirmed, killifish were injected with *A. invadans* hyphae grown in culture. The artificial infections verified the pathogenicity of the *A. invadans* culture and provided material for optimizing the assays. *A. invadans* zoospores were also assayed to show that the probe was capable of hybridizing additional life cycle stages of *A. invadans* (Fig. 2). The FISH protocol utilizes whole-cell and tissue samples and can be rapidly executed without preparing histological sections. When 50 ulcerated Atlantic menhaden collected from the Neuse and Pamlico River estuaries in North Carolina were screened, the lesions from all 50 fish were positive for *A. invadans*. A comparison of light micrographs and FISH images from each of the samples showed that all oomycete hyphae within the ulcerated tissue samples were uniformly *A. invadans* (Fig. 3). The FISH assay further confirmed that the hyphae were not merely surface contaminants but had penetrated deeply past the skin and into the muscle tissue. This work represents the first assay capable of providing rapid, species-specific, visual identification of *A. invadans* hyphae in wild-caught ulcerated fish.

The species specificity of the PCR assay developed in this study was similarly validated using a panel of oomycete cultures (Table 1). The assay was used to survey the same 50 infected fish. Each UM tissue sample tested positive for *A. invadans*. The agreement between the FISH and PCR assays indicates that PCR alone could be used as a reliable diagnostic test for the presence of *A. invadans* infection. In addition to this assay, other *A. invadans* PCR assays are available (3, 26, 38). Blazer et al. (3) used PCR to assay an unspecified number of naturally infected, formalin-preserved fish from 13 UM epidemics to demonstrate the broad geographic range of *A. invadans* extending from the Indian River in Delaware to a farm pond in Louisiana. Lilley et al. (26) tested a PCR assay on cultures of 12 *A. invadans* isolates and 12 other oomycetes to document that their assay was species specific. In a similar study, Phadee et al. (38) used a different PCR assay to screen 20 *A. invadans* isolates and >50 other isolates belonging to the Saprolegniaceae. Phadee et al. (38) also used the assay to detect *A. invadans* in experimentally challenged goldfish and estimated a lower limit of detection for the assay of ~500 fg of DNA. While the above PCR studies have provided valuable information, our work is the first large-scale sampling of a
population of naturally infected fish and is the first to demonstrate that the oomycete hyphae in the lesions were uniformly *A. invadans*. Unlike the previous studies, our study also included experimental controls that are essential for confirming the specificity and accuracy of a PCR assay. These included a PCR inhibition control to rule out the possibility of false-negative samples and a blank extraction control to identify potential DNA contamination during DNA extraction procedures.

In the past, the etiology of UM in estuaries in North Carolina, the Chesapeake Bay, and Florida has been the subject of great controversy. Initial work in the 1980s indicated that oomycetes were present in the majority of UM lesions (13, 33). Positive species identification of the oomycetes in these lesions was not possible at the time, since the necessary molecular techniques were not available, but it was hypothesized that an *Aphanomyces* species was involved in the etiology of UM (13, 33, 35). In the early to mid-1990s, such lesions were attributed to outbreaks of the putatively toxic zoospore stages of the dinoflagellate *Pfiesteria piscicida* (6–8, 14). The relationship between *Pfiesteria* and fish lesions, however, was based on laboratory studies that did not simulate natural conditions. Those studies were challenged by a number of researchers (10, 11, 41–44), and there is now a large body of research evidence that supports the primary role of *A. invadans* in causing UM.

Repeated histological surveys of UM-affected fish have found a high number of broad aseptate hyphae in the ulcers (2, 4, 12, 13, 24, 25, 35). PCR confirmed the presence of *A. invadans* in formalin-preserved ulcers (3), and laboratory exposure studies have shown that *A. invadans* zoospores can induce typical UM lesions (19, 22, 23, 27). In addition, researchers in southern Asia and Australia have identified *A. invadans* as the cause of similar ulcerative lesions in multiple fish species, including striped snakehead (*Channa striata*) and striped mullet (*Mugil cephalus*) from both freshwater and estuarine habitats (9, 15, 21, 30, 40, 45). The collective data from these studies, combined with the data from our study, support the hypothesis that *A. invadans* is the primary infectious etiological agent of UM in estuarine fish in the southeastern United States and many other regions of the world. *A. invadans*, however, should not be considered the sole etiology for ulcerative lesions. Stress, poor nutrition, and certain parasite, bacterial, and viral infections are all capable of producing similar lesions (20, 24, 32, 39).

An important question concerning *A. invadans* pathogenesis that has yet to be answered is how natural infections are initiated. Although hundreds of UM lesions have been examined, no oomycete reproductive structures have been observed (E. J. Noga, unpublished data). This observation implies that although *A. invadans* readily infects fish and often proliferates rapidly, fish are probably a dead end host. Either the infections are transferred directly between fish (less likely) or an alternate source of infection is present in the environment. Laboratory exposure studies suggest that secondary zoospores are the most likely source of UM infection (19, 22, 23). Temperature and salinity preferences, as well as sporulation cues, suggest that *A. invadans* sporulates and proliferates in low-salinity portions of estuaries following runoff events (3, 4, 13, 23, 27, 37). These low-salinity estuaries also serve as nurseries for large populations of juvenile Atlantic menhaden (1), the
most commonly infected fish in the southeastern United States. The PCR and FISH assays developed in this study now make it possible to screen the large numbers of environmental samples needed to identify alternative hosts and sources of infection for A. invadans. Identification of the infection sources and the conditions that promote growth and transmission of this pathogen will help resource managers better predict when lesion events are likely to occur and perhaps develop effective mitigation strategies.

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REFERENCES


