Advances in Detection Techniques for Fungus-like Organisms of Aquaculture Importance

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Abstract

Oomycetes are a class of eukaryotic organisms that are similar to fungus in morphology and lifestyle. The cell wall of oomycetes is composed of cellulose, unlike fungus, where chitin is the main component. These organisms are generally considered saprophytes with the ability to cause secondary infection. However, some species are highly pathogenic and cause severe diseases in fish. Important fish pathogens of this class belong to the genus *Saprolegnia*, *Achlya*, and *Aphanomyces* under the order Saprolegniales. These organisms have caused huge economic loss in aquaculture and are even considered responsible for the decline in populations of wild fish and amphibians. Previously, these organisms were effectively controlled by use of malachite green, which was later banned. This has led to the emergence of these organisms with increased incidence, virulence, and host range. The diagnosis of the disease can be made by observing the gross lesion of white cotton wool-like growth at the site of infection, but identifying a causative agent is not possible. Identification of the genus and up to the species level is made through various ways such as microscopic observation of the reproductive structures, antibody-based methods, and molecular identification techniques. With advancement in technology, rapid, specific methods have been developed that can identify as well as quantify the causative agent in a given sample. Fast and accurate identification of pathogen will enable us to act promptly against the infection to prevent further spread.

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18.1 Introduction

Fungi constitute a group of heterotrophic, eukaryotic organisms that are now considered separate kingdom different from plants and animals. Fungi are usually filamentous and multicellular, though non-filamentous and unicellular ones also exist. The filaments, also known as hyphae, are cylindrical thread-like structures, which can grow up to several centimeters. Hyphae grow at the tip and at the branches, leading to the development of a network of fungal threads known as mycelium (Fricker et al. 2007). Fungi lack chloroplast and, hence, cannot photosynthesize. They instead obtain their food from the surroundings by absorbing dissolved molecules. They can utilize almost any carbon source as food (Leaño 2001). Fungi contain chitin in addition to glucans in their cell wall. This feature differentiates the fungi from other morphologically similar fungus-like organisms such as oomycetes (water molds).

Oomycetes are a phylogenetically distinct class of organism with similar morphology and lifestyle to fungus. Previously, they were considered a fungus, but, later, they were classified as Stramenopiles and phylogenetically grouped with diatoms and brown algae (Baldauf et al. 2000; Beakes et al. 2012; Diéguez-Uribeondo et al. 2009; van West 2006). Unlike fungus, oomycetes have a cell wall, composed of cellulose (Van der Auwera et al. 1995). They rarely have septa in their hyphae or are present at the base of sporangia (Kortekamp 2005). They cause some of the most devastating diseases in plants and aquatic animals, resulting in huge economic loss and damage to natural ecosystems (Kamoun and Smart 2005; Phillips et al. 2008). Aquatic oomycetes have received lesser attention than their counterparts that infect plants (Diéguez-Uribeondo et al. 2009; van West 2006). Majority of the oomycete species that cause diseases in fish belong to the genus *Saprolegnia*, *Achlya*, and *Aphanomyces* under the order *Saprolegniales*, while a few numbers are from the genus *Pythium* (Gozlan et al. 2014). Generally, these organisms are considered secondary pathogens growing on injured or stressed fish, but some are highly virulent and can cause primary infections (Willoughby 1978; Pickering and Christie 1980). For example, *Aphanomyces invadans*, the etiological agent of the epizootic ulcerative syndrome (EUS), can cause up to 100% mortality in farmed fish (Iberahim et al. 2018). Similarly, highly virulent *Saprolegnia parasitica* has been isolated from striped catfish farms in Uttar Pradesh, India, which can cause 100% mortality in experimental infection (Ravindra et al. 2022). The genus *Achlya* also has virulent species that can cause infections in fish (Khulbe et al. 1995; Sati 1991). Some *Pythium* species were found to infect fish in natural and experimental conditions (Czeczuga et al. 2004; Khulbe 2009).

Previously, diseases caused by oomycetes were controlled effectively by using malachite green. However, this compound has been banned due to its carcinogenic and mutagenic effects (Srivastava et al. 2004). Since then, there has been a recrudescence of the disease, and new host species of the pathogens are being identified (Sarowar et al. 2013; Choi et al. 2019; Sosa et al. 2007). Infections by *Saprolegnia*, *Achlya*, and *Aphanomyces* species are often observed as a white or gray cotton wool-like appearance at the site of infection (Greeff-Laubscher et al. 2019; Mondal and De 2002; Liu et al. 2017). Thus, it is not possible to identify the causative agent by observing only the gross lesion. Therefore, to initiate effective and appropriate control measures to prevent further spread of the disease, it is crucial to correctly identify the pathogen. This will also enable the selection of appropriate chemicals and optimum doses for treatment leading to judicious use of the therapeutic compound. Requirement of drug dose may vary depending on the species, and we have also observed that different species under the same genus, *Saprolegnia*, showed variable sensitivity to antifungal compounds (unpublished data). So, species identification is essential to develop suitable programs for controlling and treating infectious agents. Identification of oomycetes is conventionally done through morphological observation, but at present, molecular techniques using DNA markers are more commonly followed.

18.2 Morphological Identification

Isolation and culture of the causative agent from the lesion or water bodies have been integral for its identification. It is a time-consuming as well as a laborious process. In our laboratory, the isolation process involved direct inoculation of the sample on potato dextrose agar (PDA) incorporated with an antibiotic (Choi et al. 2019; Parra-Laca et al. 2015; Fregeneda-Grandes et al. 2007a, b). On PDA, *Saprolegnia* and *Achlya* species produced white hyphae, some growing inside the agar and the rest extending above the surface. Each colony showing different growth morphology is further sub-cultured until a pure culture is obtained (Shin et al. 2017). Morphological identification often requires observation of different reproductive stages. In the life cycle of oomycetes, several developmental stages can be observed microscopically for identification. Their reproduction occurs sexually and asexually. In asexual mode, motile zoospores are produced and released from sporangium or zoosporangium. The zoospores swim through a pair of flagella and exhibit a chemotactic response to infect new hosts (Walker and van West 2007). In *Saprolegnia*, the primary zoospore encysts, after a short time, release the secondary zoospore, which is the most important infective stage. In *Aphanomyces*, the primary zoospore immediately encysts at the tip of hyphae; hence, the secondary zoospore is the only motile stage (Cerenius et al. 1987). In *Achlya*, it is monoplanetic, that is, the released zoospores are the only dispersal form (Daugherty et al. 1998; Johnson et al. 2002). Sexual reproduction occurs through the production of oogonia and antheridia. Structurally, antheridia are usually small and filamentous carrying nuclei containing the genome. In contrast, oogonia are large, swollen filled with oospheres, each

carrying a haploid nucleus. During fertilization, the antheridium fuses with the oogonium and releases the antheridial nuclei, which fuse with the oospheres inside the oogonium leading to the development of oospores. The oospores are spherical and double-walled and can survive in adverse environmental conditions (Gozlan et al. 2014).

Routine identification of oomycetes is made by microscopic examination of sexual reproductive structures Vandersea et al. 2006). For structural observation, oomycetes are cultured on PDA, containing sterile sesame seeds. The colonized seeds are then transferred to a new dish, containing sterile tap water and incubated for a few days. Later, the oomycetes on seeds are observed under a microscope for structures like zoosporangium, zoospores, hyphae, and sexual structures (Sandoval-Sierra and Diéguez-Uribeondo 2015). Usually, *Saprolegnia* strains, isolated from fish, do not exhibit any sexual structures in laboratory culture, and many a time, identification can be done up to genus level only (Stueland et al. 2005; Diéguez-Uribeondo et al. 2007). In addition, many species may produce similar or overlapping structures which are not stable (Diéguez-Uribeondo et al. 1996). Identification of species depending completely on morphological characteristics is not authentic and sometimes even impossible (Ke et al. 2009; Diéguez-Uribeondo et al. 2007). Instead, decorations on the secondary encysted zoospores such as long hooked hairs in bundles are considered as criteria for the identification of virulent *Saprolegnia parasitica* (Willoughby 1985; Beakes et al. 1994; Yuasa et al. 1997; Shin et al. 2017). Other saprophytic species, such as *S. diclina* and *S. ferax*, do not exhibit such characteristics (Stueland et al. 2005). In *Achlya*, the morphology of gemmae is one of the structures used for species identification. For example, spherical gemmae are a characteristic distinguishing feature of *Achlya bisexualis* (Barksdale 1962). Similarly, characteristic structures of zoospores encysted as a cluster at the orifice of zoosporangium, oogonium, and antheridium are observed to identify *Aphanomyces* species (Takuma et al. 2010). As morphological identification of oomycetes is often challenging, different detection methods based on an antibody, DNA, or RNA are being developed.

18.3 Antibody-Based Detection

The ability of antibodies to bind selectively to an antigen with high affinity has been extensively applied in many research and clinical applications. Antibodies are components of the immune system that helps the body to fight against foreign substance such as bacteria, fungi, and viruses. Antibodies may be used in diagnosis either in polyclonal or monoclonal form. Polyclonal antibodies are a mixture of antibodies that can react with a specific antigen, each identifying a different epitope (Sabeta and Ngoepe 2015). In contrast, monoclonal antibodies are derived from a single parent cell and have an affinity for the same epitope (Lipman et al. 2005). Lilley et al. (1997a, b) developed polyclonal antibodies for the detection of *A. invadans* but were found to cross react with other oomycete species. They

concluded that monoclonal antibodies (MAbs) against *A. invadans* hyphal material would provide a more specific probe for immunohistochemical detection of EUS.

Miles et al. (2003) used a monoclonal antibody against a hyphal protein of *A. invadans* for detection of the pathogen by immunofluorescent staining on tissue sections of infected fish. The antibody did cross-react with *A. astaci* but not with other oomycetes of fish. Moreover, the technique was more sensitive than the conventional methods for detection of *A. invadans*. Ganapathi et al. (2008) used monoclonal antibody (MAb)-based immunodot to screen for the presence of *A. invadans* in tissues of grossly healthy and ulcerated fishes. They found that this technique can detect the pathogen before the appearance of a lesion. They have also stated that the technique can be used for early detection of *A. invadans* and to predict EUS outbreaks at least 2 months ahead. Adil et al. (2013) have developed a monoclonal antibody-based flow-through immunoassay (FTA) to detect *A. invadans*. The assay has a detection limit of 7 μg/mL for *A. invadans*. The test is rapid, can be completed within 10 min, and is simple, cost-effective, and suitable for on-site screening to detect *A. invadans* in fish from disease outbreaks. Detection of *Saprolegnia* using monoclonal antibodies has also been reported (Bullis et al. 1996; Fregeneda-Grandes et al. 2007a, b). One group found that MAbs produced against *S. parasitica* ATCC 52719 recognized all *S. parasitica* isolates in an indirect immunofluorescence assay. Another group found variable affinity among the MAbs. They found a MAb that can recognize an epitope expressed mainly in the asexual isolates in the long-haired *S. parasitica*. They also discovered that isolates with bundles of long hairs share a number of antigens with other species of *Saprolegnia*.

18.4 Molecular Identification

In recent years, molecular detection and identification of microbes or infectious agents have become a routine work in many diagnostic laboratories. Molecular identification methods detect unique nucleic acid sequences specific to the pathogen. These techniques are more sensitive and specific than the conventional detection method. Molecular methods, owing to their high sensitivity, can detect infections at an early stage. Moreover, these methods have been successfully used for the identification of new and non-culturable agents (Morshed et al. 2007). These methods can complement the conventional method of morphological examination and serological methods for the identification of causative agents. Some highly specific methods can detect even a single nucleotide change in the nucleic acid sequence (Zaidi et al. 2003). The most powerful molecular detection method is the amplification of the target gene by polymerase chain reaction (PCR), followed by sequencing for species identification. There are other advanced forms of PCR, and many have been applied in the detection and identification of oomycete species. In addition, molecular methods do not require amplification of the target gene or nucleic acid but use a complementary probe, for example, fluorescent in situ hybridization (FISH). The types of molecular techniques developed and used for detecting and identifying fish oomycete pathogens are discussed below.

18.4.1 PCR and Sequencing

Amplification of the internal transcribed spacer (ITS) region, followed by sequencing, is commonly done to identify oomycete species. The ITS region is routinely amplified because its copy number is high, enabling detection even in a small quantity of DNA, it is small in size, it has high variation even between closely related species, and it is flanked by highly conserved sequences (Baldwin et al. 1995). The ITS region is situated between 18S and 26S gene and includes ITS1 and ITS2 separated by 5.8S gene in the nrDNA. Amplification of ITS region is done by using universal primers, developed by White et al. (1990). In our study, ITS1 (forward, 5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (reverse, 5′--TCCTCCGCTTATTGATATGC-3′) primers have been used. In *Saprolegnia* and *Achlya*, these primers yielded a PCR product of 750 bp approximately. The PCR products were cleaned and subjected to Sanger sequencing for species identification. The sequence is then compared with the available nucleotide sequences in the database through Basic Local Alignment Search Tool (BLAST), and species was identified. Based on the sequences of the ITS region, 29 DNA-based molecular operational taxonomic units (MOTUs) were identified, which supported the validity of 18 species of *Saprolegnia* and 11 potentially new species (Sandoval-Sierra et al. 2014). Another commonly targeted gene for the identification of oomycetes species is cytochrome *c* oxidase subunit I (COI), which is a relatively new approach. Amplification of the ITS region uses genomic DNA, whereas mitochondrial DNA is used for COI. It has been reported that COI, in some case, is more delimiting than ITS at the species level (Robideau et al. 2011).

18.4.2 Identification Without Sequencing

Nucleotide sequencing is the process of determining the order of four nucleotides in nucleic acid. Identification of species by Sanger sequencing is time-consuming, and a higher cost is involved as often the service needs to be outsourced. Therefore, different types of PCR for species identification without sequencing have been developed or applied. Random amplification of polymorphic DNA (RAPD) PCR was used to analyze the genetic diversity in *Aphanomyces astaci*, the etiologic agent of crayfish plague, and to trace the origin of outbreaks (Huang et al. 1994; Lilley et al. 1997a, b). Bangyeekhun et al. (2001) applied RAPD-PCR to characterize *Saprolegnia*, isolated from catfish, and confirmed the presence of three genetically distinct groups. In RAPD PCR, arbitrary primers are used to amplify random segments of the target gene. When separated in a gel, the PCR products give a distinct DNA fingerprint.

Another technique that has been used for species identification in oomycetes is restriction fragment length polymorphism (RFLP). It is a technique in which one or more restriction endonucleases digest the DNA sample, and the digested fragments are separated in gel electrophoresis. The DNA fragments are then transferred to the membrane through Southern blotting. Then the RFLP probe is allowed to hybridize

with the DNA fragments, giving a unique blotting pattern characteristic of a particular species. Since the isolation of enough DNA for RFLP is time-consuming and laborious, PCR amplification is performed before digestion with restriction enzymes. Molina et al. (1995) used restriction fragment length polymorphisms (RFLPs) to characterize and identify *Saprolegnia*. They subjected amplified products of the ITS region to a number of restriction endonucleases to generate several fingerprints. They found that endonuclease BstUI generates identical fingerprints for all strains of *S. parasitica*, regardless of its origin. Like RFLP, amplified fragment length polymorphism (AFLP) also uses restriction enzymes. It is a highly sensitive and reproducible technique. In this technique, genomic DNA is first digested, and adaptors are ligated to the sticky ends of the fragments. Then selective amplification of the fragments using primers complementary to the adaptor and restriction site is carried out. The amplicons are separated on denaturing gel, and the band pattern is visualized (Vos et al. 1995). Rezinciuc et al. (2014) stated that AFLP is an alternative method to RAPD-PCR for genotyping *A. astaci*. Elameen et al. (2021) used AFLP technique to study the genetic diversity and relationships of *Saprolegnia* spp. collected from different geographical locations. They found that AFLP analysis has a significant correlation with ITS sequence data.

Significant development of PCR technology is real-time PCR, also referred to as quantitative PCR. As the name indicates, this method detects and measures the generated products in real time. Here, the detection of the product can be achieved in two ways: (1) use of a non-specific fluorescent dye that intercalates with double-stranded DNA and emits fluorescence and (2) use of a sequence-specific probe labelled with fluorescent reporter, which emits fluorescence when cleaved by *Taq*DNA polymerase during extension of primer toward the probe. The intensity of the fluorescence is measured with a detector corresponding to the increase in the amplified product (Heid et al. 1996). Rocchi et al. (2017) developed a real-time quantitative PCR (qPCR) to quantify *S. parasitica* in a river as well as drinking water. Di Domenico et al. (2021) developed real-time PCR TaqMan assays to distinguish the five genotype groups of *A. astaci.* They stated that the technique is suitable for fast genotyping of *A. astaci* during crayfish plague outbreaks and in latent infections. Ghosh et al. (2021) have developed loop-mediated isothermal amplification (LAMP) to detect *Saprolegnia* species. The detection method targeted the ITS region and COI gene and was specific only to *Saprolegnia* genus. The method is highly sensitive, with a detection limit of 10 fg of DNA.

DNA probes labelled with a fluorescent dye are also used in the molecular cytogenetic technique known as fluorescence in situ hybridization (FISH) for species identification (Frickmann et al. 2017). This method can detect specific nucleic acid targets in cells and tissue samples. In this assay, the probe is allowed to hybridize to its complementary sequence in the denatured DNA present in the specimen. The hybridization between the probe and the target DNA can be visualized using a fluorescent microscope. Vandersea et al. (2006) have developed fluorescent peptide nucleic acid in situ hybridization (FISH) assays to detect *A. invadans* in ulcerated lesions. They found that the results of the assay exactly matched with PCR targeting the region containing the 18S gene and ITS1.

18.4.3 Visual Detection

Parra-Laca et al. (2015) designed a PCR-free *Saprolegnia* detection kit using hemolymph from the adult female insect *Dactylopius coccus*. They conceptualized the idea based on the reports that the immune components of the insect react with the fungal cell wall component such as N-acetylglucosamine and 1–3 glucan and form melanin, which can be observed visually. Their study found that *Saprolegnia* sp.-induced reaction was utilizing the pigment carminic acid of the insect hemolymph. Consequently, there was a formation of melanin which could be observed visually, and the presence of *Saprolegnia* was identified. The assay is capable of identifying the presence of *Saprolegnia* between 5 and 282 zoospores.

18.5 Conclusion

Fish disease, caused by oomycetes, is fast emerging, as there is no effective treatment against these pathogens. The disease is no longer confined to only freshwater fish. It has also been reported from marine species. This class of organism is generally considered secondary pathogens that infect only when there are injury or primary infections. However, some species are highly virulent and can cause huge mortality in farmed fish. Moreover, the new hosts of the pathogens are being discovered. Therefore, it is important to delineate the pathogenic species from the non-pathogenic ones. There have been many developments in the detection and identification methods of oomycetes, but point-of-care diagnostic system is lacking. Future research may aim to develop a rapid, easy, or user-friendly identification system having field application. This will help in early diagnosis at the farm level, and immediate necessary measures can be taken to prevent further spread of the disease.

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