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Report of *Saprolegnia salmonis* infection in ornamental fish

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Abstract

Saprolegniosis poses a significant challenge in the aquaculture industry, leading to substantial losses, including an estimated one egg loss for every thirty eggs in cold-water fish hatcheries. The *Saprolegnia* genus, classified under the Saprolegniaceae family, comprises water molds that thrive in freshwater environments. They are recognized as a critical fungal group affecting both farmed and wild fish.This fungus is naturally found in fresh water and moist soil and is responsible for Saprolegniosis. Recently, several ornamental koi fish exhibiting symptoms such as white patches, weakness, lethargy, skin lesions, fin deterioration, and swimming incoordination were referred to the microbiology department at the Faculty of Veterinary Medicine, University of Tehran. Initially, the symptoms raised suspicions of Cort syndrome; however, further testing confirmed Saprolegniosis. Skin samples from the affected fish were collected and cultured on Sabouraud dextrose agar (SDA) at 20°C. Phenotypic methods were used to determine the genus of the fungus, followed by genotypic methods, specifically polymerase chain reaction (PCR), for species identification. The PCR product was sequenced by Sina Clone Company and deposited in a gene bank, ultimately confirming the presence of *Saprolegnia salmonis.* Given the disease's direct correlation with the fish's living conditions, owners were advised to improve water quality, ensure appropriate living conditions, and manage dietary needs. Additionally, enhancing public awareness of Saprolegniosis and improving hygiene standards in endemic areas is recommended to mitigate its impact on the aquaculture sector.

Introduction

Saprolegniosis is one of the most devastating diseases affecting freshwater fish, caused by species of the genus *Saprolegnia*, which includes 23 or more species (Elameen *et al*., 2021). This disease is characterized by clinical signs such as visible white or gray patches of filamentous mycelium on the skin and fins. In severe cases, the fungal hyphae invade the epidermal tissues, muscles, and blood vessels, ultimately leading to the death of infected fish due to osmoregulatory failure (Elameen *et al*., 2021). The genus *Saprolegnia* contains significant pathogens in aquaculture, although it is often considered an opportunistic pathogen that is both saprotrophic and necrotrophic (Elameen *et al*., 2021). However, some strains, particularly *Saprolegnia parasitica*, are highly dangerous and can cause primary infections (Elameen *et al*., 2021). Therefore, distinguishing these pathogenic species from non-pathogenic ones is essential. Several techniques, including microscopic examination of mycelia, sexual reproductive structures, and oospores, were the primary methods used for species identification until molecular techniques were

developed (Ghiasi *et al.,* 2014).

Some species are found in brackish waters, but salinity levels exceeding 2.8% limit the spread of these fungi; therefore, the greatest damage caused by these fungi occur in freshwater environments with fish adapted to these conditions (Ghosh *et al.,* 2021). Species of *Saprolegnia* affect not only various cultivated fish species, such as rainbow trout, atlantic salmon (*Salmo salar*), salmon, catfish, and major carp species, including Indian rohu, Catla, and Black carp, but also impact populations of mollusks, amphibians, and wild fish. Notably, this impact can manifest at all stages of the fish life cycle, including in fish eggs (Shahbazian *et al.,* 2010; Adel *et al.,* 2020).

In the past, malachite green was used to treat saprolegniosis; however, due to its carcinogenic and toxic effects, its use in aquaculture has been prohibited since 2002, leading to the resurgence of various *Saprolegnia* species, including *S. salmonis* (Ghiasi *et al.,* 2014). Despite the correlation of saprolegniosis occurrence with certain water parameters such as temperature, pH, ionic concentration, and organic content, systematic protocols for managing *Saprolegnia* remain absent. Furthermore, considering the importance of tropical aquaculture systems in Asia, which account for 80% of aquaculture production, early detection of *Saprolegnia* could significantly reduce the loss of infected fish and the resultant economic losses (Adel *et al.,* 2020, Tedesco *et al.,* 2021).

Typical conditions in salmonid farming environments, such as high biomass density, frequent handling of fish for grading and reproduction, and other predisposing factors, can increase the likelihood of *Saprolegnia* infections through enhanced spore release into the aquatic environment (Najafipoor, 2011). It is worth noting that one of the main issues related to these intensive aquaculture systems is the proliferation of parasites, especially those with direct life cycles, and other infectious agents due to the high density of cultivation, which can subsequently spread to the natural environment (Adel *et al.,* 2020, Tedesco *et al.,* 2021). Therefore, the risk of disease transmission from wild fish to farmed fish, followed by further proliferation in the environment and transfer to ecosystems, could pose a concerning future challenge. Although limited studies on this fungus have been conducted in various regions of Iran, including Mazandaran, Kermanshah, and Golestan provinces, it can be said that the ecology of *Saprolegnia* species is one of the least studied aspects of the genus, not only in Iran but also in other countries (Shahbazian *et al*., 2010). In this report, we described saprolegniosis caused by *S. salmonis* in several ornamental koi fish in the microbiology department of the Faculty of Veterinary Medicine at University of Tehran.

Materials and Methods

Several ornamental koi fish exhibiting signs of weakness, anorexia, lethargy, swimming incoordination, and mortality were referred to the microbiology department of the Faculty of Veterinary Medicine at University of Tehran. Essential information was obtained regarding the water parameters of the ponds, including temperature, salinity, oxygen levels, and pH, reported in degrees Celsius, percentage (%), mg/l, and so forth.

Clinical examinations revealed reports of white hyphae on the body (Fig. 1), skin lesions, fin deterioration, and in some samples with more severe symptoms, invasion of the hyphae into the epidermis, muscles, and blood vessels, which in some cases led to osmotic failure in the fish. To identify the fungi phenotypically, the samples were cultured on Sabouraud dextrose agar (SDA), and then the samples were thoroughly examined under a microscope. Under microscopic observation, the mycelium and hyphae of the fungi, along with sexual reproductive structures, were identifiable (Figs. 2, a and b). Subsequently, the polymerase chain reaction (PCR) method for molecular analysis and species determination was employed.

After definitive identification, the fish were treated with procaine baths, administered at a dose of 2-5 mg/kg depending on the fish's weight and the severity of the disease, for an average of one to three weeks. Signs of improvement gradually appeared after two weeks.

Materials

DNA Extraction Kit: Sinagen (Iran), Specific Primers: SinaClon (Iran), Agarose Gel: 1.5% (Sigma, USA), Sabouraud Dextrose Agar: Merck (Germany) (SDA), Master Mix: Ampliqon (Denmark)

Phenotypic identification

The identification of *Saprolegnia salmonis* was conducted based on its macroscopic and microscopic characteristics through cultivation in SDA at 23°C for five days. Fungal staining was performed using Giemsa staining (Arda, 2006) to facilitate the identification of

fungal samples. Identification was based on the criteria established by Dvarak and Atanoesk (1969).

Sampling

In May 2023, 10 samples suspected of having saprolegniosis were collected and referred to the microbiology laboratory at University of Tehran. A total of 10 fish, with an approximate weight of 800 g, underwent clinical and molecular examinations.

Testing method

To analyze the genotypic characteristics of the identified fungi and for final confirmation, the PCR test was conducted using specific forward (ITS-1-F) and reverse (ITS-4-R) primers (SinaClon Co., Iran) to amplify the ITS1-5.8S rDNA-ITS2 region (Table 1). DNA extraction was performed using the Sinagen DNA extraction kit (Sinagen Co., Iran). The quality and quantity of the extracted DNA were assessed spectrophotometrically at a wavelength of 260 nm. The extracted DNA samples were stored at -20°C until PCR analysis.

For the PCR test, the optimal concentration of materials used in the PCR reaction was prepared in a total volume of 25 µl for each tube, consisting of 2.5 µl of 10X PCR buffer + MgSO4, 1 µl of each primer (at a concentration of 10 picomoles), 0.5 µl of a dNTP mix (at a concentration of 0.2 millimolar), one unit of Pfu DNA polymerase, and 1 µl of fungal DNA (at a concentration of 100 nanograms) (Table 2). The PCR cycles included an initial denaturation at 94°C for 90 seconds, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 68°C for two minutes, with a final extension of 10 minutes at 68°C (Table 3), using a Thermocycler (Biorad Co., USA). The PCR product was purified and recovered using a PCR purification kit. The recovered product was then sequenced by TAG Copenhagen. Following local alignment (BLAST), the sequences were deposited in the GenBank database.

Electrophoresis of the PCR products was conducted using an electrophoresis apparatus (PadidehNojen Pars Co., Iran) on 1% agarose gel (Sigma Co., USA) stained with Safe Stain (SinaClon Co., Iran). The PCR reaction products were purified and recovered using a PCR product purification kit (Qiagen Co., Iran). The recovered product was then sequenced by TAG Copenhagen (Denmark), and after local alignment (BLAST), finally it was registered in the GenBank database.

Results

The primers used in this study were capable of accurately identifying the ITS-1 region in *S. salmonis*. The PCR was employed to amplify the aforementioned region, resulting in the generation of a fragment approximately 550 base pairs in size (Fig. 3).

Table 1. Primers for *Cyprinus carpio* Gene for detection of saprolegniosis (Sangalang *et al.,* 1995, Taylor *et al.,* 2000).

Target	Primer	Sequence	Size (bp)
ITS-1	ITS-1-F	5'-TCCGTAGGTGAACCTGCGC-3'	550
	ITS-4-R	5'-GCATCGATGAAGAAGAACGCAGC-3'	

Table 2. Materials required for PCR reaction.

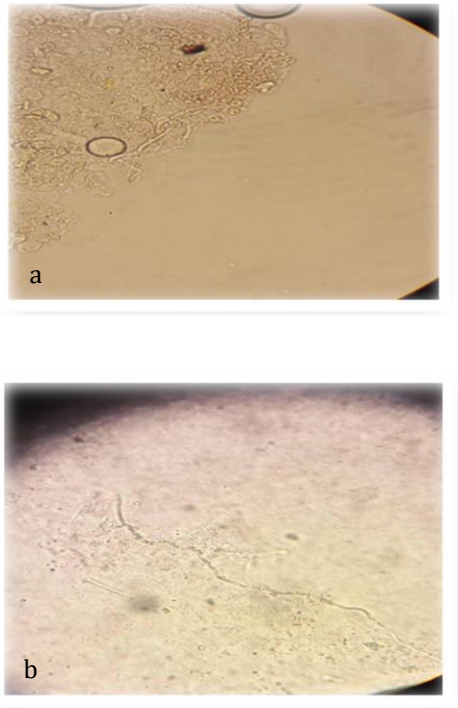
Materials	Reaction Volume (µl)	Final Concentration
Primer F	1	10 pmol
Primer R	1	10 pmol
PCR Master Mix	12.5	1x
DNA	1	100 ng
H_2O	9.5	-

Table 3. Thermal cycling conditions for PCR reaction.

Step	Number of cycles	Temperature (°C)	Time
Initial Denaturation	1	94	90 Sec
Denaturation	-	94	30 Sec
Annealing	40	60	30 Sec
Extension	-	68	30 Sec
Final Extension	1	68	10 Min


a

Fig. 1. White to gray cotton-like patches on the skin and gills.



Figs. 2 (a and b). Mycelium, sexual reproductive structures, and oospores observed under the microscope.

Fig. 3. Lane M: 1000 bp marker; Lane 1: positive sample with a specific band for the ITS-1 gene at 550 bp.

Discussion

Saprolegniosis is a global disease and an acute infection affecting a wide range of freshwater fish and their eggs, caused by certain species of *Saprolegnia*. While its presence in fish does not directly impact humans, it can lead to secondary infections or allergic sensitivities. Despite the economic importance of saprolegniosis, which significantly affects a large portion of the aquaculture industry, detailed information on the pathogenic mechanisms and host responses to species such as *S. salmonis* is still lacking.

In the current case study, a brief report was presented on saprolegniosis caused by *S. salmonis* in several koi ornamental fish at the Faculty of Veterinary Medicine at the University of Tehran. Considering that the Persian sturgeon is the most important native sturgeon species in the southern Caspian Sea (Mirmazloomi *et al.,* 2022), any environmental pollution or disease can affect its reproduction and migration (Mirmazloomi *et al.,* 2022). Unfortunately, in Iran, despite the need to focus on this fish species alongside reports indicating annual declines, comprehensive studies on *Saprolegnia* species in sturgeon have not been conducted (Mirmazloomi *et al.,* 2022).

Among the studies conducted in Iran, a molecular study in Mazandaran province on the eggs of Persian sturgeon *(Acipenser persicus)* reported the presence of *Saprolegnia* species in all examined samples (Shahbazian *et al.,* 2010, Najafipoor, 2011). Another study in Mazandaran indicated that *S. parasitica* was the primary cause of fungal attacks on salmonid eggs, accounting for 13.18% of infections. Most studies increased following the work of Jalilpour *et al*. (2006), which reported a mortality rate of 7% to 22% of *Acipenser* eggs during a collective incubation at a specialized fish farming center in Iran (Masigol *et al.,* 2021).

Results from studies conducted in other countries similarly showed that saprolegniosis is the most problematic infection for salmonids in freshwater farms in Japan. Annual losses in trout farming businesses in Scotland, Scandinavia, Chile, Japan, Canada, and the United States are estimated to be in the tens of millions of pounds (Elameen *et al.,* 2021).

Therefore, further studies are needed considering the significance of the mentioned aquatic species. In this study, saprolegniosis was diagnosed using phenotypic methods, followed by confirmation through PCR testing. This report emphasizes the need for more molecular research on aquatic organisms, especially freshwater fish and Persian sturgeon in Iran.

Given that this type of fungus affects nearly all freshwater fish and can be transmitted to the environment through wild fish and even amphibians

(Elameen *et al.,* 2021; Chanu *et al.,* 2022), early and frequent removal of infected fish from reservoirs and the artificial breeding of sturgeon from the Caspian sea is conducted to preserve their populations and for preventive measures (Tedesco *et al.,* 2021).

Additionally, it is worth noting that the global growth of aquaculture reflects a change in our dietary habits. Fish farming has significantly increased in recent years and is on the verge of surpassing beef as a food source by the end of this decade (Van West, 2006). Over the past century, the world has heavily relied on two natural systems: ocean fishing and cattle farming to meet the increasing demand for animal protein. However, both systems are now reaching their production limits (Van West, 2006). Aquaculture, particularly the farming of rainbow trout, has developed in Iran and has become an important economic industry. However, recent reports indicated that about half of the produced trout eggs are lost due to fungal infections (Shahbazian *et al*., 2010; Ghiasi *et al.,* 2014).

Currently, one of the potential solutions for controlling saprolegniosis in aquaculture systems involves the development of vaccines against *Saprolegnia*. Several laboratories are conducting various studies to utilize vaccines against *Saprolegnia* as a model. Ultimately, further studies are recommended to gain more information in ecological, pathogenic, and vaccination domains.

In conclusion, saprolegniosis represents a serious fungal infection in freshwater fish and poses a significant threat to the aquaculture industry, resulting in considerable economic losses. Conducting advanced molecular research and developing effective vaccines could provide a viable solution for controlling this disease and reducing associated mortality in fish farming systems (Van West, 2006). Moreover, more comprehensive studies on pathogenic mechanisms and host immune responses could enhance prevention and treatment strategies.

In this study, after collecting 10 samples of fish suspected of having saprolegniosis and conducting preliminary phenotypic examinations, including culture on SDA and observation of macroscopic and microscopic characteristics, an initial diagnosis was made. To confirm the diagnosis, the PCR method utilizing specific primers for ITS1-5.8SrDNA-ITS2 was employed. DNA extraction was performed using the Sinagen kit, and the quality of the DNA was validated via spectrophotometry. Following PCR amplification and electrophoresis of the products, 8 out of the 10 samples examined were definitively identified as infected with *Saprolegnia* fungus. These results indicated a high prevalence of saprolegniosis in the studied fish population.

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Conflict of Interest

No conflict of interest has been reported among the authors.

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