Microscopic and molecular investigation of the parasite *Ichthyophthirius multifiliis* in rainbow trout in the fish breeding ponds of Rijab city.

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Abstract

Introduction: Rainbow trout is the most important farmed species of cold-water fish that is densely raised in the country's farms. Parasitic contamination is one of the factors that can endanger aquatic production. The parasite *Ichthyophthirius multifiliis* is one of the most dangerous external parasites of fish, especially juvenile freshwater fish. *I. multifiliis* is a ciliated protozoan that causes white spot disease. The aim of this study was to determine microscopically and molecularly the presence of *I. multifiliis* in rainbow trout from the Rajab fish breeding ponds.

Materials and Methods: This research was conducted in the city of Rajab during the spring season of 1402 (2023). A total of 100 rainbow trout samples were collected from several different fish breeding ponds and euthanized using MS-222. Samples consisted of skin scrapings and skin mucus, which were stored in a freezer at -20°C. To investigate the prevalence and perform microscopic and molecular evaluation of *I. multifiliis*, PCR was used. The accuracy of PCR detection was verified by sequencing, and finally a phylogenetic tree was constructed for the studied sequences.

Results: Microscopic examination using the wet mount method revealed ciliated parasites with horseshoe-shaped nuclei, which were morphologically similar to *I. multifiliis*. The PCR test estimated a prevalence of 20%. Sequencing results showed that the amplified DNA sequences were completely identical to data recorded in the NCBI database. The studied sequences exhibited high genetic similarity to each other and, in descending order, were similar to isolates Ark10, Ark5, and NY4. The sequences originated from a single population or species.

Conclusion: Due to the potential for parasitic infections and other pathogens associated with the presence of native fish (acting as susceptible hosts or reservoirs) in the river and upstream fish farms, and given the relatively high prevalence of *I. multifiliis* of foreign origin, compliance with health protocols in rainbow trout farms is essential to prevent the introduction of parasitic pathogens.

Keywords: I.multifiliis parasite, microscopic evaluation, PCR, phylogenic tree, prevalence.

1- Introduction

The parasite *Ichthyophthirius multifiliis* is considered one of the most dangerous external parasites of fish, especially juvenile freshwater fish. With the expansion of the fish farming industry and increased stocking densities, this parasite has spread widely due to the increased likelihood of the free-living stages of the parasite encountering new hosts in intensive farming conditions [1].

I. multifiliis is a ciliated protozoan that can cause "white spot disease," a condition that poses significant challenges to aquaculture and aquarium fish producers worldwide. The parasite can produce numerous protozoa through simple cell division, potentially leading to thousands of mature parasites. The disease is highly contagious and can be exacerbated under high fish density. An outbreak of *Ich* in fish farms is considered an emergency, requiring immediate treatment. Delayed treatment can result in 100% mortality of the affected fish [2].

White spot disease, or ichthyophthiriasis, is one of the most common and significant parasitic diseases in the aquaculture industry [3]. This disease is somewhat seasonal due to temperature conditions, often occurring in spring and autumn, with some epizootics observed in winter [4]. Severe infections may occur on the gills with little evidence of infection on the skin [5].

In a 2013 study at the Aquatic Animal Health and Diseases Research Center of the Faculty of Veterinary Medicine, University of Tehran, Zargar et al. reported that *I. multifiliis* had the highest infection rate, while *Chilodonella* had the lowest [6].

Azargoon and colleagues conducted a study in 2013 on *I. multifiliis* in rainbow trout in Ramsar, Iran, during the winter and summer seasons. They found that the prevalence of this parasite was 26% in summer and 19% in winter [1].

In 2008, Kabirian et al. identified the concurrent presence of *I. multifiliis* and the bacterium *Aeromonas hydrophila* as the cause of severe mortality in rainbow trout farms in the suburbs of Mashhad (Friz village) [6].

In a 2022 study on rainbow trout, Gökmen and colleagues aimed to calculate the overall prevalence of *Ich* and identify new *Ich* genotypes. They found an overall prevalence of 35.3% and described five new *Ich* genotypes based on mitochondrial genes COX1 and nad1-b. Their phylogenetic analysis also revealed that Turkish *Ich* strains are genetically divided into at least four distinct groups [7].

Claire et al. (2017) conducted a study on rainbow trout in the Klamath River, California, to monitor the parasite *I. multifiliis*. Their results demonstrated that PCR analysis of environmental water samples could serve as a valuable surveillance tool for detecting the presence of *Ich* in the Klamath River [8].

Mallick et al. (2015) investigated the occurrence of *I. multifiliis* infection in rainbow trout, revealing that the infection pattern was temperature-dependent, with the highest prevalence observed in July [9].

Jørgensen et al. (2009) conducted a study aimed at determining parasitic infections in rainbow trout (*Oncorhynchus mykiss*) farms. This study examined parasitic infections in restored rainbow

trout farms over 22 months. Parameters such as temperature, mortality, pH, nitrite and ammonia concentrations, formalin usage, mortality, and feed conversion ratio were also assessed. The study found that, following the introduction of rainbow trout from traditional earthen ponds to new systems, all farms were infected with several known parasites from traditional farming practices. In some farms, white spot disease caused by *I. multifiliis* was associated with high fish mortality. The findings indicated that seasonal fluctuations in parasite populations were somewhat masked by the effects of chemical treatments and the less variable temperature levels throughout the year [10].

The objective of this study is to investigate the parasite *I. multifiliis* in rainbow trout farming ponds in Rijab city, with the hypothesis that *Ichthyophthirius* infection is present in rainbow trout in the Rijab region and that PCR is a definitive method for detecting this parasite.

2- Materials and Methods

This study was conducted in the city of Rijab during the spring of 2023. A total of 100 rainbow trout were sampled from various fish farming ponds. The fish were caught using a dip net and euthanized with MS-222 without being washed to ensure that parasites adhering to the surface of the body were detected. The samples consisted of skin scrapings and mucus. The samples were stored at -20°C.

DNA extraction from the samples was performed using the Favorgen commercial kit. Favorgen is a commercial kit designed for DNA extraction from various tissues and biological materials, based on a microfluidic column-assisted extraction method. The steps for DNA extraction are as follows:

1. **Preparation**: All surfaces and equipment were sterilized with isopropyl alcohol solution before starting.
2. **Sample Processing**: A 25 mg tissue sample was placed in a sterile microtube, and 200 µL of FATG1 buffer was added. The sample was mixed thoroughly using a pipette.
3. **Incubation**: The microtube was incubated at 60°C for 1 to 3 hours to allow the tissue to lyse completely, with intermittent vortexing.
4. **Buffer Addition**: After incubation, 200 µL of FATG2 buffer was added to the microtube, mixed thoroughly, and incubated at 70°C for 10 minutes.
5. **Ethanol Addition**: Next, 200 µL of 96-100% ethanol was added and mixed. The sample was then centrifuged at 18,000 rpm for 1 minute.
6. **Column Binding**: The supernatant was carefully transferred to a column placed in a collection microtube.
7. **Washing**: The column was washed with 400 µL of W1 buffer and centrifuged at 18,000 rpm for 1 minute, after which the collected liquid was discarded. This washing step was repeated with an additional 400 µL of buffer, followed by centrifugation and disposal of the collected liquid.
8. **Drying**: The column was centrifuged at 18,000 rpm for 3 minutes to dry.
9. **Elution**: 100 µL of preheated Elution buffer (70°C) was added to the column, incubated for 3 minutes, and then centrifuged at 18,000 rpm for 2 minutes to elute the DNA.

The concentration of the extracted DNA was measured using a NanoDrop spectrophotometer. Additionally, to assess the purity of the DNA, absorbance readings were taken at wavelengths of 280, 260, and 230 nm using a spectrophotometer.

primer design was carried out using Oligo7 software and the Primer3 website. The following key points were considered in primer design:

- **Primer length:** Primer lengths were set between 18 and 25 base pairs.
- **GC content:** The GC content of the primers was set between 40 and 60%.
- **Melting temperature:** The melting temperature of the primers was selected so that the primers would melt at a suitable temperature for the PCR reaction.
- **Specificity:** The primers were designed to specifically bind to the target sequence and amplify a product of approximately 530 base pairs.

Ultimately, the following primers were designed for this study:

- Forward primer: 5'-ATGATTTCTATGCCTGCAAC-3'
- Reverse primer: 5'AAAGTAGATGAAGTGTGAAGTCT-3'

These primers were successfully used to amplify the desired DNA fragment. These primers were ordered from Gene SinaClon Company. The primers were then dissolved in sterile distilled water and their final concentration was adjusted to 100 picomoles per microliter.

Samples were packaged for shipment in sterile vials, under completely sterile conditions and at a temperature of 4 degrees Celsius for the shipment of biological samples.

To perform PCR on the samples, PCR was carried out in a thermocycler using the following temperature program: First, the sample was placed at 94 degrees Celsius for 3 minutes. In the second step, the temperature was set at 94 degrees Celsius for 30 seconds. In the third step, the temperature was decreased to 55 degrees Celsius for 30 seconds, and in the fourth step, it was maintained at 72 degrees Celsius for 30 seconds. Then, steps 2 to 5 were repeated 35 times. Finally, the sample was placed at 72 degrees Celsius for 10 minutes.

In the next step, a 1% agarose gel was prepared and the PCR product was loaded into it. Then, the gel was run on an electrophoresis apparatus at a voltage of 100 volts for 30 minutes. After the electrophoresis was completed, the gel was visualized under UV light using a transilluminator. In samples suspected of having Ich parasites, if a PCR product with a size of about 530 base pairs was observed, it indicated the presence of Ich parasites in the samples.

3- Result

In the microscopic examination using the wet mount method, samples of the fish's skin and gills were placed on a slide and prepared with normal saline solution to maintain a moist environment. Under the microscope, ciliated parasites with a horseshoe-shaped nucleus were observed, resembling the parasite *Ichthyophthirius multifiliis* (Figure 1).

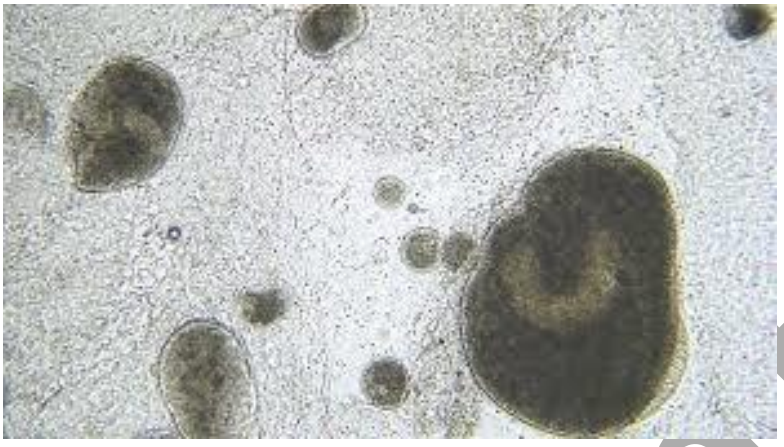

Figure 1: Under the microscope, ciliated parasites with a horseshoe-shaped nucleus were observed. These parasites resembled I. multifiliis.

The presence of *Ichthyophthirius* in the studied fish indicates a potential risk of white spot disease in these specimens.

In the PCR assay, a specific 530 nanometer DNA fragment was amplified using specific primers. Gel image analysis revealed a band of 530 base pairs in 10 wells. This band was clearly visible, indicating the presence of the target DNA in these 10 samples. Wells 1 to 7 and 9 to 11 contained samples with the target DNA. Well 8 contained a 100bp ladder used for sizing the band. Well 12 contained a negative control to ensure no contamination of the experiment. Well 13 contained a positive control to ensure the proper functioning of the assay. Based on the experimental results, it can be concluded that 20 out of 100 tested samples contained the target DNA. This result indicates a high prevalence (20%) of the target DNA in this population.

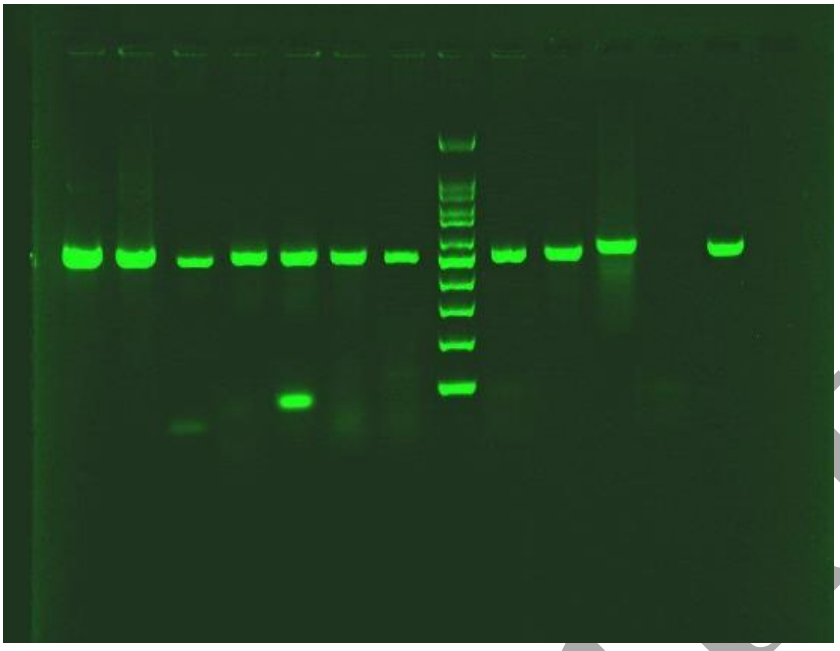

Figure 2: Gel electrophoresis results for the evaluation of samples and controls. Gel image analysis revealed a band of 530 base pairs in 10 wells.

Referring to Figure 2, from left to right, wells 1 to 7 contain the samples being tested. Well 8 contains the 100bp ladder, and wells 9 to 11 contain the samples being tested. Well 12 is the negative control, and well 13 is the positive control.

the accuracy of PCR detection was verified using sequencing techniques. For this purpose, DNA samples were extracted, and then the PCR technique was used to amplify a region of the DNA. Finally, sequencing techniques were used to determine the sequence of the amplified DNA. The results obtained from sequencing showed that the sequence of the amplified DNA was completely identical to the data recorded in the NCBI database. This indicates that the results obtained from PCR were completely accurate.

the MEGA 11 software was used to investigate the genetic relatedness of the detected strains using the pairwise distance parameter. Then, the maximum composite likelihood method was used to estimate similarity, and the bootstrap method was used to estimate the statistical distribution and account for uncertainty. This method showed that the sequences under study were very similar in terms of genetic similarity and, in the next step, were similar to isolates Ark10, Ark5, and NY4, respectively. The least similarity was observed with isolate BR1. It seems that the sequences under study originated from a single population or species. This result was expected given the very high similarity of these sequences.

the following phylogenetic tree was drawn for the studied sequences. For this purpose, the MEGA 11 software and the Neighbor-joining method were used. The bootstrap method was also used to estimate the statistical distribution and account for uncertainty (Figure 3).

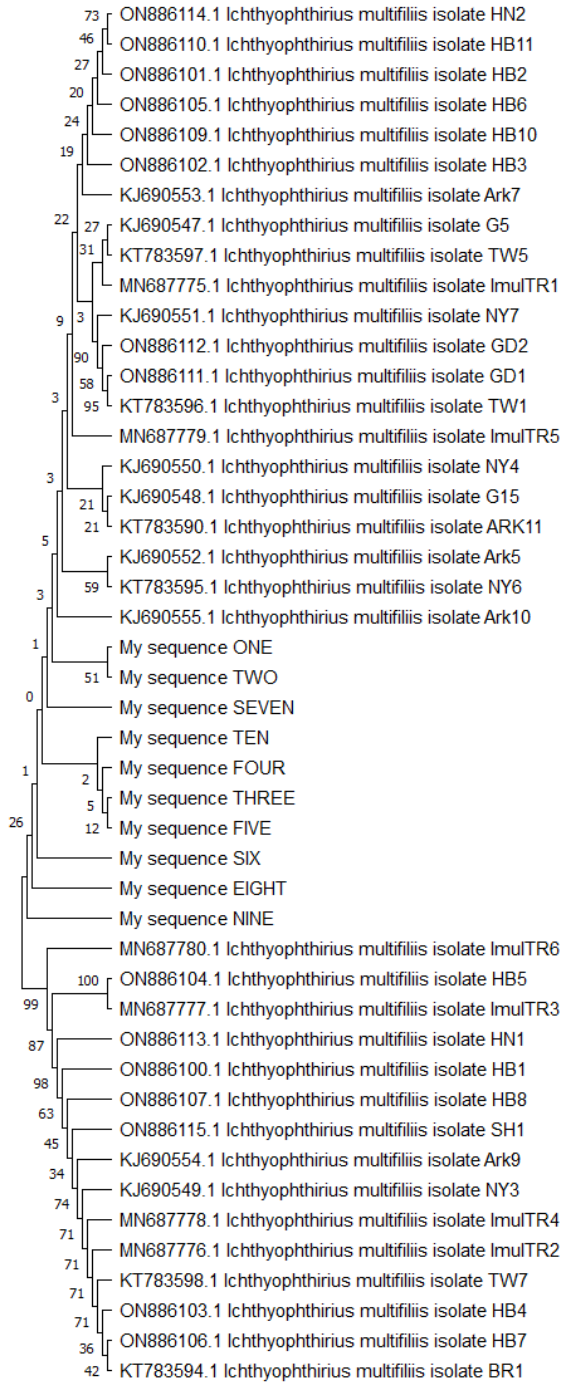

Figure 3: The phylogenetic tree shows the studied sequences, labeled as "My sequence 1" through "My sequence 10".

As observed, the studied isolates have clustered into similar groups. This finding is consistent with the results of the genetic relatedness analysis. Overall, the results of this study indicate that the studied sequences originated from a single population and are more closely related to isolates Ark10, Ark5, and NY4.

4- Discussion

Undoubtedly, issues related to fish contamination, especially parasitic infections, are of significant importance for the health, production, and quality in rainbow trout aquaculture facilities. Therefore, attention to hygiene and pathogenic factors that can affect fish stocking density is crucial. Various researchers in recent years have investigated parasitic infections in this valuable fish, particularly focusing on the transmission of parasites from natural water sources to aquaculture farms. This study aimed to identify and investigate the prevalence of *I. multifiliis* in farmed rainbow trout in the Rijab region.

The parasite *I. multifiliis* is primarily an external parasite of the skin and gills of freshwater fish, capable of causing severe infections and high mortality rates. The host range of this parasite is broad, and under favorable conditions regarding density and temperature, it can rapidly proliferate in farms, leading to white spot disease. The life cycle of this parasite is temperature-dependent. During the growth phase in the host tissue, trophonts can exceed one millimeter, with increased capillary disruption and necrotic changes. The parasite feeds on cellular components and creates small cavities beneath the epithelial layer [11]. Upon exiting the skin and gills, the remaining lesions disrupt osmotic balance and lead to the host's death. This parasite can infect fish at all ages and easily transfers from natural water sources to farms, as well as through the movement of fish between farms. Studies have shown that native fish from springs, qanats, and rivers are major reservoirs of this parasite and can serve as principal vectors during the warmer seasons. In fish hatcheries, especially in recirculating water systems with high fish densities and multiple water uses, the parasite can cause devastating outbreaks [12].

Microscopic examination in the present study using wet mount preparation revealed ciliated parasites with a horseshoe-shaped nucleus, resembling *I. multifiliis*. The presence of this parasite in the studied fish indicates a probable infection with white spot disease. PCR results showed a 20% prevalence of *I. multifiliis* in rainbow trout from the Rijab region. [3] reported a 36.66% prevalence of *I. multifiliis* in a fish health research center at the University of Tehran, which is consistent with our study. [1] reported varying prevalence rates of *I. multifiliis* at 26% in summer and 19% in winter. Considering that water temperatures in Rijab's trout farms are also low, the prevalence observed in our study aligns with [1] findings.

he results of the study by [13] reported a prevalence of *I. multifiliis* in rainbow trout in West Azerbaijan at 11.26%. Their studies also indicated that river water, due to the presence of non-cultured fish and relatively unstable temperature, is a source of contamination for rainbow trout farms. Considering that the water source for the Rijab region's ponds is also the Alvand River, the prevalence in Rijab's trout farms is consistent with [13] findings.

The physiology, environmental tolerances, and biological variations of the parasite suggest the existence of multiple strains. Traditionally, strains are described by serotype, which is defined by the combination of antigenic i on the surface of *I. multifiliis*. This serological classification has limitations, as it does not reveal phylogenetic relationships or provide insights into valid genotypes or molecular diversity of the parasite. Additionally, different serological isolates of *I. multifiliis* are closer to each other than to isolates of the same serotype. Our study results showed that the sequences studied were highly similar to each other and were most closely related to the strains Ark10, Ark5, and NY4, with the least similarity to the BR1 strain. It appears that the sequences studied originate from a single population or species. This result was expected given

the high similarity among these sequences. However, the phylogenetic analysis by [7] revealed that Turkish *I. multifiliis* strains are genetically divided into at least four distinct groups. Additionally, five new genotypes of *I. multifiliis* were described based on mitochondrial genes COX1 and nad1-b. A NY3 strain and five new strains were identified in Turkish *I. multifiliis* isolates based on COX1 and nad1-b sequences.

Determining the genetic diversity of field isolates of *I. multifiliis* can help explain the relationship between *I. multifiliis* prevalence and their potential genotypes, and enable tracking of pathogen genotypes worldwide [7].

5- Conclusion

Considering the potential for parasitic infections and other pathogenic factors due to the presence of native fish (as sensitive hosts or reservoirs) in rivers and upstream fish farms, and given the relatively high prevalence of *I. multifiliis* of external origin, maintaining hygienic practices in rainbow trout farms is crucial for preventing the introduction of parasitic pathogens. It is recommended that future studies investigate additional parameters such as fish age, environmental temperature, and physical and chemical parameters of the farming environment. Furthermore, future research should evaluate the prevalence of *I. multifiliis* in farms where the water is sourced from springs and wells.

Ethics approval

This study was conducted following ethical guidelines for animal research. The fish samples were handled humanely, and euthanasia was performed using MS-222 to minimize suffering. The research protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of [Islamic Azad University, Sanandaj , Iran]. All procedures conformed to the standards for the ethical treatment of animals in research.

Author Contributions

C.A.K. conceived the idea, designed the experiments, and conducted the study.. assisted in sample collection and performed PCR analyses. B.S. contributed to the data analysis and interpretation of results. S.K. helped with sample preparation and sequencing. C.A.K. led the manuscript preparation. All authors critically reviewed the manuscript and approved the final

Conflict of Interest

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Data Availability

The DNA sequences generated and analyzed during this study are available from the corresponding author upon reasonable request. The sequence data have not been deposited in public repositories but can be provided for verification purposes. All other data supporting the findings of this study are included within the article.

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