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PCR Based Identification of *Saprolegnia parasitica* Affecting Rainbow Trout Aquaculture in Kashmir Region of Indian Himalayas

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ABSTRACT

Saprolegnia parasitica infecting Rainbow trout (*Oncorhynchus mykiss*), has been a major bottleneck in the intensification and propagation of trout culture in Northern Himalayas of India. Present study was to ascertain the presence of *S. parasitica* infections using molecular diagnostic techniques in cultured rainbow trouts collected from different hatcheries of Kashmir valley. Growth of *S. parasitica* at different incubation temperatures was examined. The molecular detection of fungus done by using PCR, revealed that the twenty trout specimens out of 144 collected samples were found infected with *Saprolegnia parasitica* with overall prevalence of 13.88%, with a PCR product size of around 750 bp. No growth of *S. parasitica* was reported at 4°C after 48 h of incubation. *S. parasitica* infections were more frequent in cold water months when water temperature was recorded between 7°C - 9°C.

Key words: Kashmir, PCR, Saprolegnia, Trout.

INTRODUCTION

Rainbow trout (*Oncorhynchus mykiss*) is a cold water exotic species, which thrives naturally in lotic systems and has carnivorous mode of feeding. Among all oily fishes, trout contains lowest amount of dioxin (2,3,7,8-Tetrachlorodibenzo-p-dioxi)- an environmental contaminant. Farmed rainbow trout is low in mercury and polychlorinated biphenyls (PCBs). The fish is preferred nutritionally since it is rich in proteins, omega-3 fatty acids, potassium, phosphorus, vitamin-B complex and selenium. But in Kashmir valley temperate conditions pave favourable pathways for fungal infections to attack in captive as well as wild stocks, thereby leading to the decline in their crop. Although regarded as the secondary infections, fungal diseases are more prone in Kashmir trout farms and known to cause huge economic losses to the sector. The primary factors responsible for flourishing fungal disease in the fish hosts include deprivation of temperature and dissolved oxygen, high salinity and overcrowding of fishes. The fishes already infected with pathogens such as virus and bacteria are also prone to the attack by fungal infections and easily fall their prey.

Among fungi, *Saprolegnia* is the main genus causing significant infections in fresh water fishes both in hatcheries and growth-out farms. The disease condition is responsible for significent economic losses across the globe. In the economic importance, fungal infections are second only to bacterial diseases (Hussain *et al.,* 2001) around the world, among which *S. parasitica* has been mostly found to affect fisheries sector. The fungus is characterized by having non-septate, multinucleate hyphae, with profusely branched mycelium that can easily be identified on substrate. Sometimes hyphae penetrate deep inside the muscles and blood vessels (Hatai and Hoshiai, 1992). The disease is characterized by the growth of whitish fungal mesh on the epidermal tissues of the infected sites of fish; usually skin (Dermatomycosis) and gills (Branchiomycosis). This gives the appearance of cotton wool, hence this disease is also known as 'cotton wool disease'. Saprolegnia eventually causes hemodilution i.e; osmoregulatory failure (Hatai and Hoshiai, 1994) resulting in fish death. The chances of Saprolegniasis are high in colder months (Mastan and Ahmad, 2018), infections usually occur at low water temperatures (8.5°C -11.5°C) and automatically vanishes as temperature rises above 11.5°C (Shah *et al*., 2015).

Saprolegnia species have been mainly identified on the basis of their morphological characters of the sexual reproductive structures such as oogonia, oospores and antheridia (Leclerc *et al.,*2000; Dick, 1969). However, several species show similar characters and also their morphology is not stable and constant (Liu *et al.,* 2017). Moreover, the fungus collected from animals often fails to form sexual structures in-vitro conditions (Dieguez-Uribeondo *et al*., 2007). Therefore, traditional morphological criteria do not

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provide a strong basis for the identification and classification of *Saprolegnia* (Liu *et al,* 2017; Dieguez-Uribeondo *et al.,* 2007). Molecular approaches such as PCR amplification and sequencing of the internal transcribed spacer (ITS) region of ribosomal RNA (rRNA) genes are thus some of the basic molecular diagnostic tools which can be helpful in identifying these organisms. It not only helps in precise identification of organisms but also provides a basis for their treatment as well.

The present study was undertaken to assess and diagnose disease condition caused by *S. parasitica* in trout farms of Kashmir using PCR technique. The study is first of its kind in Kashmir scenario and will be helpful in its managmnet. It will pave way to devise the treatment plans to protect the crop at the earliest.

MATERIALS AND METHODS

Study area and collection of fish samples

Infected rainbow trout having whitish growth of fungal mesh on the epidermal tissues were collected from 10 different trout farms from eight districts of Kashmir during 2018-19. Each specimen was throughly examined for disease condition. Every effort was made for safe transportation of infected fish specimens to the Aquatic Animal Health Management Laboratory, Faculty of Fisheries SKUAST-K. A total of 144 fish samples were taken.

Isolation of Saprolegnia species (Shah *et al.,* 2015)

Wet mounts of fungal mycelium taken from the skin of infected live trout fish specimens were cultured on Sabouraud Dextrose Agar (SDA) culture plates. With the help of a sterile platinum loop of wire, some bits of fungal mycelia were taken and streaked on to the surface of plated semi-solid media. The plates were incubated at 30±2°C for 7 days.

Purification of Saprolegnia isolates

After 7 days, the germinated mass along with the bit of media was transferred on to the fresh plate of agar and kept for incubation (Shah *et al,* 2015; Tiffney, 1939; Raper, 1937). After the appearance of a definite mycelium colony around the inoculation site, a second block of agar about one square centimetre was cut from the edge of the colony and placed on the inoculated surface downwards on to a third plate of agar. The same procedure was repeated five times till bacteria free isolates were obtained.

Preparation of fungal slides

Slides were prepared using Lactophenol Cotton Blue (LCB). The slides were examined under a microscope for studying the fungal morphology (Thomas *et al*., 1991).

Fungal DNA extraction and its visualization (Gardes and Bruns 1993)

The obtained fungal mass from culture plates was homogenized in tissue homogenizer twice using lysis buffer (100 mM Tris HCl [pH 8.0], 50mM EDTA, 3% SDS). The homogenate obtained was centrifuged at 13,000 x g for 10 min. The supernatant was transferred to a fresh microcentrifuge tube. Two micro-liters of RNase A (10mg/ml) was added to supernatant and the sample was incubated at 37°C for 15 min. After that equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added, content was mixed well and then centrifuged at 13,000 x g for 10 min. To the upper aqueous layer equal volume of 100% ethanol was added in new centrifuge tube. DNA was allowed to precipitate at -20°C for 30 min. To pellet down the DNA, the whole content was again centrifuged at 12,000 x g for 10 min. The DNA pellet obtained was washed with 70% ethanol and centrifuged at 12,000 x g for 5 min. After that the pellet was air dried and dissolved in 1× TE buffer. Electrophoresis was carried on 1% agarose gel in 1X Tris-acetate-EDTA buffer, using ethidium bromide (2 µl). Bands were visualized under UV transilluminator and results were recorded by photography.

Concentration and Purity of extracted DNA

Nanodrop was used for quantification and purity check of the extracted fungal DNA. Two micro-liters of extracted DNA was kept in the nanodrop and the DNA concentration was estimated. Ratio between absorbance at 260nm/ 280nm gave the purity ratio.

Polymerase Chain Reaction

Primers used for detecting *Saprolegnia parasitica* Internal Transcribed Spacer (ITS) genes *viz*., (ITS_1 and ITS_4) were diluted with nuclease free water. Primers specific to ITS1 (Forward: 5′ -TCCGTAGGTGAACCTGCGG-3′) and ITS4 (Reverse: 5′ -TCCTCCGCTTATTGATATGC-3′) genes having gene bank accession code number (AY455771) were procured from ThermoScientific, USA. PCR reactions were performed in reaction tubes containing 12.5 µl of Green Master Mix (2x), 2 µl of each primer (10 µM), 5 µl of DNA template and 3.5 µl of DNase free water to obtain final volume of 25 µl (Touhali, 2018). The amplifications were carried out in Eppendorf′s gradient thermal Master cycler with initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, primer annealing at 58°C for 30 s and extension at 72°C for 1 min. The final extension step of 5 min at 72°C was performed (Touhali, 2018). The product (750 bp) was stored at 4°C. The resultant PCR products were subjected to electrophorosis on1% agarose gel in 1X Tris- acetate-EDTA buffer, using ethidium bromide (2 µl). Bands were visualized under UV transilluminator and results were recorded by photography.

Growth of S. parasitica at different temperatures

Disks were punched at the centre of SDA plates (diameter of disk = 6 mm). The isolated *Saprolegnia* was placed in wells, and the plates were placed at different temperatures, 4°C, 20°C and 30°C for 48 h for incubation. Colony growth on SDA was measured using Vernier calliper.

Statistical analysis

The correlation between the fungal infections and the water temperature was calculated using Pearson's rank correlation.

RESULTS AND DISCUSSION

Fishes with or without scales are equally susceptible to fungal infections. Fungal species namely, *S. diclina, S.ferax, S.hypogyana, S. parasitica* and *Achyla americana* are pathogenic to fishes (Mastan, 2015). Among fungal infestations in fish, *S. parasitica* is highly detrimental (Scott and O. Bier, 1962). *S. parasitica*poses a serious threat to aquaculture systems (Molina *et al.,* 1995; van West 2006; Phillips *et al.* 2008), affecting both brood fish and incubating eggs. Saprolegniasis usually appears as a secondary infection to the fish integument already damaged because of some other infections. Sometimes, *Saprolegnia* can act as a primary pathogen infecting fish that have no signs of previous damage (Verma, 2008). Typically, saprolegniosis does not usually penetrate deep into muscle but the lesions are seen confined to the skin surface. The severity of the disease is determined by the area covered by the damage. Infected fish show whitish to greyish cotton like growth on the skin which is a typical clinical sign of Saprolegniosis (Eissa *et al*, 2013; Touhali, 2018). Same observations were recorded in the rainbow trout during the present study (Fig 2). Pale and necrotic gills were also observed in infected fishes. Water pollution and overcrowding enhance the disease occurrence. Fungal infection caused by *S. parasitica* causes high mortalities in Salmon culture systems, worldwide (Hatai and Hoshai, 1992). Around 10% of salmon hatchlings die due to the disease, causing huge loss to fish industry, which is around

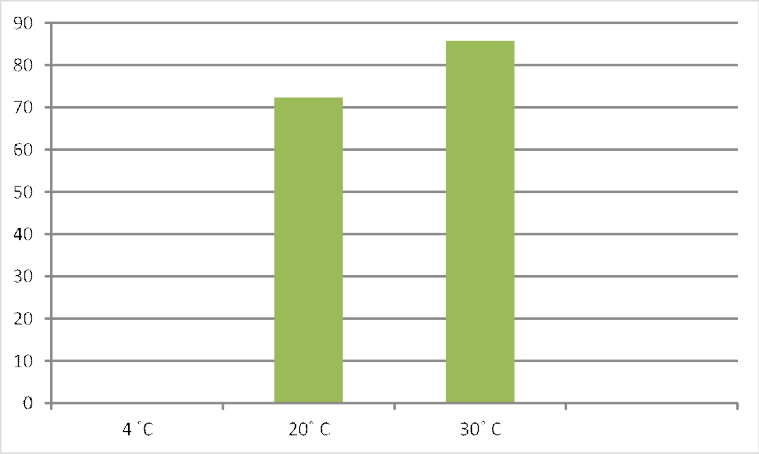

Fig 1: Diameter of fungal colony (mm) at different temperatures.

Table 1: Month-wise prevalence of *S. parasitica.*

Months	Temperature °C	No. of suspected specimens examined	*S. parasitica* infected specimens	Frequency of infection
December	8°C	12	4	33.33%
January	7°C	12	6	50%
February	9°C	12	3	25%
March	13°C	12	2	16.66%
April	18°C	12	1	8.33%
May	21°C	12	0	0%
June	24°C	12	0	0%
July	30°C	12	0	0%
August	29°C	12	0	0%
September	21°C	12	0	0%
October	17°C	12	2	16.66%
November	7°C	12	2	16.66%

Table 2: Correlation between water temperature and incidence of *S. parasitica* infestations.

		Temp.	Fungus infected
Temp.	Pearson Correlation	1	-.838**
	Sig. (2-tailed)		.001
Fungus infected	Pearson Correlation	-.838**	1
	Sig. (2-tailed)	.001	

**Correlation is significant at the 0.01 level (2-tailed).

30% of the global fish production (Molina *et al.,* 1995; Murray and Peeler, 2005; van West, 2006; Fregeneda-Grandes *et al.,* 2007; Phillips *et al.,* 2008).

Saprolegniosis is more prevalent at cold water temperature (Mastan and Ahmad, 2018; Shah *et al*., 2015) which is in agreement with the findings of the present study. In the present study, 20 trout specimens out of 144 collected samples were found infected with *Saprolegnia parasitica* with overall prevalence of 13.88%. The highest frequency of disease occurrence was found in January (50%) followed by 33.33% infected subjects in December. Presence of fungal strain was not found in summer months while on the onset of autumn, the infections were again found raised to 16.66% in October and November (Table 1 and Fig 1). Thus, there was a significant correlation (P≤0.01) found in the occurrence of *Saprolegnia* infections and variations in the water temperature (Table 2 and 3). The infection was found to be more frequent during winter months (December-February) when water temperature ranged between 7°C – 9°C.This study further revealed that dense fungal growth occurs at 30°C culture occupying whole plate, on the other hand it cannot grow at 4°C after 48 h of incubation, as earlier reported by Liu *et al*, (2017), who observed fewer incidence of Saprolegniosis, at water temperature is less than 10°C. Pure colonies of *S. parasitica* on SDA after 24-48 h of incubation at 30±2°C appeared as white cotton tufts which gradually turned brownish after storage for two weeks (Fig 2). The observed colour change in the fungal colonies was in accord with earlier findings of Touhali, (2018). In the current study, microscopic examination of fungus stained with Lacto phenol cotton blue revealed non-septate

Table 3: Relationship between temperature and incidence of *S. parasitica* infestations.

	Mean	Std. Deviation	N
Temp.	17.0000	8.29019	12
Fungus infected	1.6667	1.92275	12

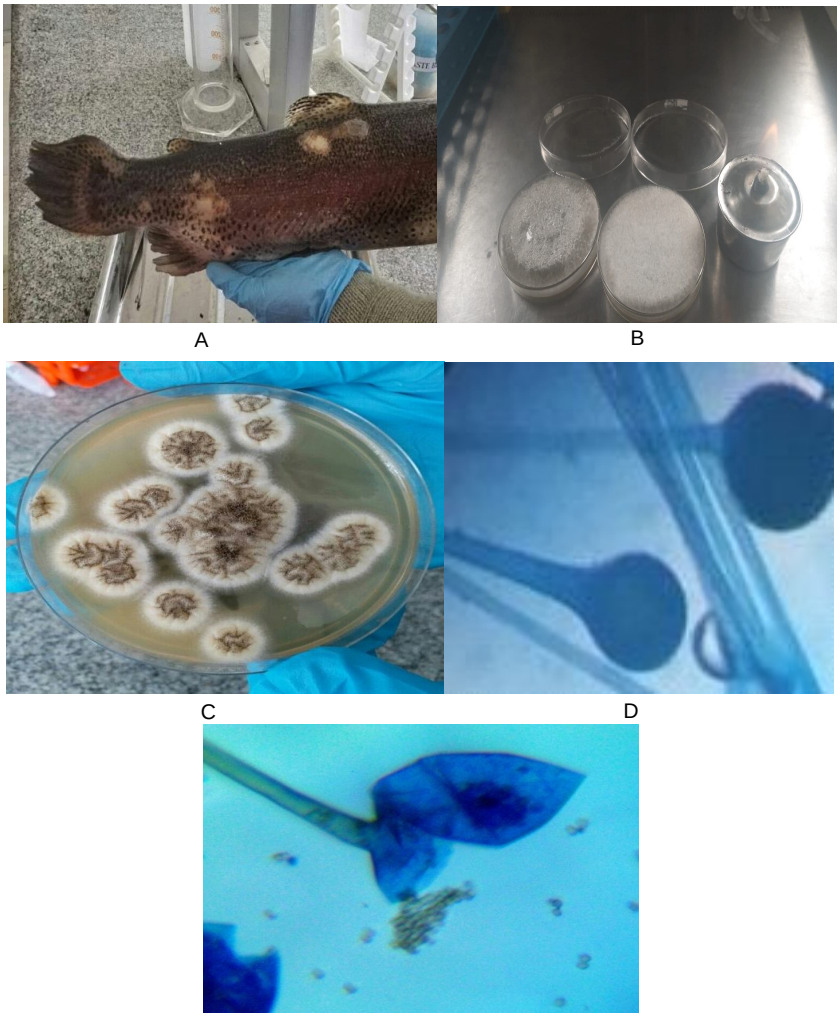

Fig 2: A- Cotton like patches on infected specimens. B- White colonies of *S. parasitica* on SDA. C- Turning of white colonies into brownish-black after 2 weeks. D- Non-septate mycelium of *Saprolegnia parasitica* with round oogonia at tips. E- Release of oospores from oogonium.

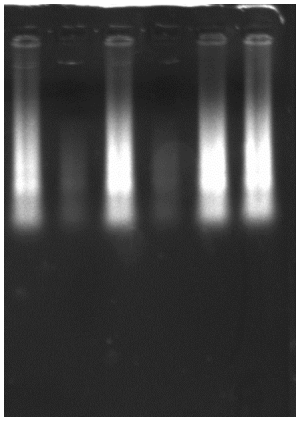

Fig 3: Isolated DNA from *S. parasitica.*

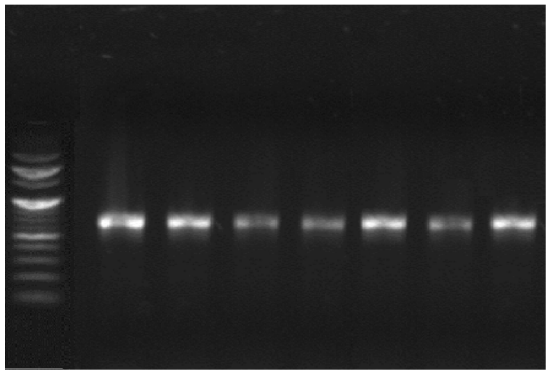

Fig 4: Amplified PCR product on agarose gel electrophoresis: Lane 1: 100bp DNA ladder; Lane 2: Positive control of *S. parasitica*; Lane 3-8: Test samples for *S. parasitica* (750bp).

multinucleate profusely branched mycelium with roundish oogonia at the ends of fungal hyphae (Fig 2) which was in agreement with previous studies (Touhali 2018; Daugherty *et al*., 1998; Shah *et al.,* 2015).

Concentration of DNA extracted from fungus (Fig 3) estimated using Nanodrop (ThermoFisher, USA) was found 115.3 ng μL^{-1} while as the purity ratio was found 1.93. In the present study, polymerase chain reaction amplifications of internal transcribed spacer genes: ITS_1 and ITS_4 showed clear bands corresponding to 750 bp, confirming the presence of *S. parasitica* in infected trout (Fig 4). Similar PCR results were conveyed by earlier studies (Eissa *et al.*, 2013; Sherif and Abdel-Hakim, 2016; Touhali, 2018; Wang *et al.,* 2019).

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