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Full Length Research Paper

Freshwater fungi isolated from eggs and broodstocks with an emphasis on *Saprolegnia* in rainbow trout farms in west Iran

Firooz Fadaeifard[1]*, Mehdi raissy[1], Hamidreza Bahrami[2], Ebrahim Rahimi[3] and Ahmad Najafipoor[4]

[1]Department of Aquatic Health and Disease, Shahrekord Branch, Islamic Azad University-Shahrekord-Iran.
[2]Department of Microbiology, Shahrekord Branch, Islamic Azad University-Shahrekord-Iran.
[3]Department of Food Hygiene, Shahrekord Branch, Islamic Azad University-Shahrekord-Iran.
[4]Faculty of Veterinary Medicine, Shahrekord Branch, Islamic Azad University-Shahrekord-Iran.

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Fungal diseases of fresh water eggs and fishes are known to be a problematic disease. The aim of this study was to investigate the aquatic fungal flora specially oomycete fungi in rainbow trout hatcheries from west part of Iran during fall and winter 2008, an attempt to isolate fungi from the eggs and broodstocks showing fungal infection at the three rainbow trout farms. A total of 150 randomly selected eggs and 15 broodstocks were obtained. A sterile swab was taken from outer surface of body (skin, gills, fins), as well as eggs. sabouraud dextrose agar, glucose yeast agar and Hemp seed in sterile tap water was used for fungi isolation. Identification of the fungi was based on their vegetative organs, including hyphae shape and size, asexual reproduction organs, shape of sporangium and spores, and generative organs, structure of oogonium, oosporangium and antheridium. In this study eight genus and species were identified and the most common were *Penecillium* sp., *Acreomonium* sp., *Alternaria* sp., *Fusarium solani*, *Aspergillus* sp., *Mucor* sp., *Saprolegnia* sp. and *Cladosporium* sp. Among the above species *Penecillium* sp. with 23% and *Saprolegnia* sp. with 3% have more and less occurrence, respectively. Identification of *Saprolegnia* sp. which is an important pathogen in aquaculture needs further study in the future.

Key word: Fungal infection, fish, *Saprolegnia*, Iran.

INTRODUCTION

Among numerous aquatic fungus species Oomycetes have special importance because of their effect on fish health and the following economical losses (West, 2006). Czeczuga et al. (2004) isolated 15 fungal species such as *Aphanomyces frigidophilus* and *Saprolegnia salmonis* from eggs of *Coregonus lavaretus* holsatus. More than 80 isolates from different kinds of aquatic fungus belong to *Saprolegnia*, *Pythium, Thraustotheca*, *Achlya*, *Aphanomyces*, *Dictyuchus* and *Protachlya* which identified as special parasites of temperate fish in india. Between all of these *Saprolegnia* and *Achlya* was the most virolent parasite in comparison to the others (Sati, 1991). Firoozbakhsh et al. (2005) for the first time were reported as isolation of *Branchiomyces*, *Phoma* and *Exophiala* from *Cyprinus carpio*, *Hypophtalmichthys molitrix* and *Ctenopharingodon idella* in north Iran. Ebrahimzadeh et al. (2007) with study on the fungal infection of rainbow trout eggs in Mazandaran Province farms of Iran indicated that *Saprolegnia parasitica* was the most important fungal egg infestation in Mazandaran salmonid hatcheries. Pickering and Willoughby (1977) by studying epidermal lesions of fungal infected perch showed that these lesions contain as many as four different aquatic fungi growing together as a mixed colony. A total of six genera of freshwater Oomycetes *(Achlya, Aphanomyces, Leptolegnia, Leptomitus, Pythiopsis* and *Saprolegnia)* have been isolated from a

*Corresponding author. E-mail: fadaeifard@gmail.com. Tel/Fax: 0098-381-3361045.

fungal infected perch in Windermere.

Saprolegniasis is a continuing problem for aquatic animal culturists. *S. parasitica, Saprolegnia diclina* and *Saprolegnia ferax* are known to be pathogens to cold water fishes. Any species of fish that is intensively cultured and captured, is at risk of contracting fungal diseases (Blazer et al., 2002). Saprolegniasis is the most troublemaking infection of salmonids in fresh water farms of japan (Hussein et al., 2001). Chukanhom et al. (2004) identified fungal infections of common carp (*Cyprinus carpio*) eggs in Thailand and succeed to isolate some fungal agents like *Allomyces arbuscula* for first time. Following the occurrence of mortality in farmed salmonids of Japan, 33 strains isolated from injuries on the basis of their morphological and biological characteristics and with transferring in the hemp seed cultures had placed in Saprolegnia class. 15 strains as a *S. parasitica*, 16 strains as a *Saprolegnia salmonis* and 2 strains *Saprolegnia australis* had identified (Hussein et al., 2001). Saprolegniasis epizootics in Coho salmon (*Oncorhynchus kisutch*) 20 to 60 g in size occurred in Prefecture Miyagi (Japan) that in the clinical signs and isolating of pathogenic fungus, *S. parasitica* distinguished as a cause of disease (Hatai and Hoshina, 1992).

In Iran, there has been an increase in the production and consumption of freshwater fish and shrimp reared in aquaculture systems in recent years, mainly the rainbow trout (*Oncorhynchus mykiss*). In this research we attempt to isolate and identify the fungal species infecting rainbow trout broodstocks and their eggs by standard morphological methods.

MATERIALS AND METHODS

Collection of fish and egg samples

Collection of fish and egg samples was accomplished during spawning season, autumn and winter 2008. A total of 15 and 150 samples of rainbow trout brood stock and eggs were collected alive from three hatcheries in west part of Iran, respectively. In each farm 50 eggs were collected with sterilized forceps from different incubator and transferred to a screw cap tube which has been sterilized with distilled water. These randomly chosen samples were transported alive to the mycological research laboratory.

Isolation of zoosporic fungi

For the isolation of zoosporic fungi associated with fish and egg, samples of gills, fins, skin and eggs were cultured in sabouraud dextrose agar (SDA) and Saprolegniaceae specific medium, glucose-yeast extract (GY) agar. The GY agar consist of 10 g of glucose, 2.5 g yeast extract and 15 g agar in 1,000 ml distilled water (Hatai and Egusa, 1979). To inhibit the bacterial growth, 500 µg/ml each of penicillin and streptomycin was added to the medium. The isolates were incubated at 15°C on GY agar, and transferred to fresh GY agar monthly. For purification, grown fungi were transferred to fresh medium. Isolates had studied by macroscopically (colony shape and color, way of growing) and microscopically (existing septate wall, sexual organ structure, size and arrangement of its spores) examinations.

In order to stimulate the Saprolegniaceae to make sexual organs head part of each colony in addition to the part of medium had cut in approximate dimensions in 5 mm and transferred to the plate which has a hemp seed in sterile tap water (Johnson, 1956). After inoculation, hemp seed cultures were incubated at 10 and 20°C and mediums evaluate daily for fungal growth. Finally, the isolated strains were identified according to Willoughby (1978), Von Arx (1981) and Howard (1983).

RESULTS AND DISCUSSION

In present study eight fungal species, *Penecillium* sp., *Acreomonium* sp., *Alternaria* sp., *Fusarium solani*, *Aspergillus* sp., *Mucor* sp., *Saprolegnia* sp. and *Cladosporium* sp. were isolated from rainbow trout eggs and broodstocks. Identification of isolates was accomplished on the basis of their vegetative, asexual reproduction and generative organs. *Saprolegnia* sp. according to thick hypha and cylindrical zoosporangium in shape and spheroid cyst distinguished and fungal colonies were seen as a white and cotton shape on the glucose-yeast agar which filled the entire medium after about a week. Mycelia as a thick, aseptate with 30 to 75 micron in diameter observed that also hyphae with fewer diameters observed too. The highest infection with 45% was found in farm A and the lowest with 20% in farm C. The most occurrence of fungal isolates belong to *Penicillum* sp. and the fewest were found in *Saprolegnia* sp. (Figure 1). Diversity of fungal isolates according to selected farms is seen in Table 1.

The aims of this study were to survey the diversity of fungal species isolated from eggs and broodstocks of rainbow trout fish in the west of Iran. Nowadays fungal infections and subsequent damages and mortalities are the most effective causes which result in an economical loss in rainbow trout farming. Especially in the present of fungi Oomycetes which play an important role among the family of Saprolegniaseae (Neish, 1977). With regard to the role of them in the involving broodstocks and eggs, they can have direct impact on the production rates and economical conditions of farms, so paying attention on environmental and management conditions, which reduce these infections, is necessary.

Relative frequency of identified fungi in different farms shows that farm A with isolating 7 strains of fungus and 46/6% relative frequency has high fungal infection in the period of sampling and low in farm C with 10%, although these differences are not significant. Increase or decrease in infection rate of hatcheries depend on management circumstances and conditions of broodstock fishes so that the following should be paid attention to: using healthy broodstocks without any external injuries, preventing from stress in incubation period , disinfection of troughs and hatchery water with using the suitable disinfectants, observing the egg reap process and keeping the fertile eggs in suitable condition. The interaction of physicochemical factors generally influence on the diversity of water molds (Paliwal et al., 2009).

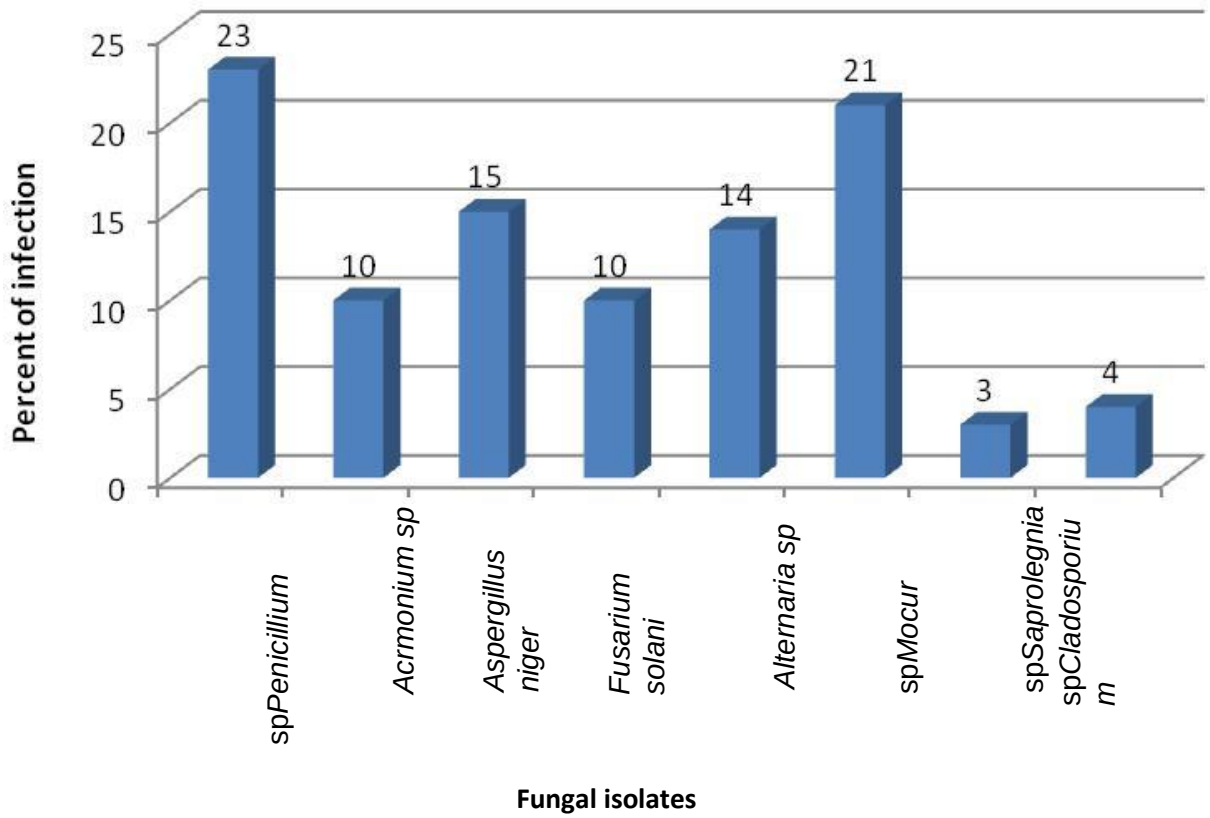

Figure 1. Distribution of relative frequency of identified fungi in eggs and fish.

Table 1. Diversity of zoosporic fungi isolated from fish and eggs samples in the different farms.

Farm / Fungi	Farm A	Farm B	Farm C
Penicillium sp.	+	+	+
Acremonium sp.	-	+	-
Aspergillus niger	+	+	-
Fusarium solani	+	-	+
Alternaria sp.	+	-	-
Mocur sp.	+	+	+
Saprolegnia sp.	+	-	-
Cladosporium sp.	+	+	-
Total	7	5	3

One of the fungal samples in present study was *Aspergilus niger* that leads to internal and external infection in fish. Involvement rate of this fungi was 10% of total infection and also in Iran from the environment of water, outer surface of fishes' body and fish eggs had been reported (Firoozbakhsh et al. , 2005; Ebrahimzadeh et al., 2007). The most abundant strain in this study is *Penicilium* which also in most resources count as a ubiquitous fungi in nature but did not isolate from fishes as a pathogenic agent, some types of *Penicilium* are able to make pathological signs in fish. Mycotic infections associated with Saprolegniacea are widely reported in fresh water fish. They are rarely found in brackish water (Hussein et al., 2002).

It is proven that ecological differences in different geographical locations play an important role in the species diversity of the fungi that develop on both fish and eggs (Wood and Willoughby, 1986). Although

environmental variables were not studied in this study, they are known to influence the growth, reproduction, and intensity of aquatic fungal infections. In addition, the occurrence of fungal infections may be related to environmental changes or seasonal variations, water quality, temperature as well as physiological changes and the immune response of fish. According to Willoughby (1994) fungi which belong to genus of Saprolegnia can be one of fungal causes in fresh water fishes and their eggs. The results of present study from the point of relative frequency confirm this.

New rRNA-targeting oligonucleotide probes permitted the fluorescence *in situ* hybridization (FISH) identification of freshwater fungi in an Austrian second-order alpine stream. Based on computer-assisted comparative sequence analysis, nine taxon-specific probes were designed and evaluated by whole-fungus hybridizations (fish) is a reliable tool for the detection of growing stages and the spatial distribution of aquatic hyphomycetes in lotic systems (Baschien et al., 2008).

This suggests that among all kinds of fungal infections, Saporlegnia in rainbow trout eggs has a lot of importance (Hussein et al., 2001). In a study by Kiziewicz (2004), aquatic fungal pathogens include *Saprolegnia ferax, S. monoica, S. parasitica, Achlya debaryana, Aphanomyces leavis, A. polyandra, Dictyuchus monosporus, D. sterile* and human fungal pathogens were isolated.

Barnes and Gaikowski (2004) with survey on ability of controlling fungal infection of Chinook salmon, *Oncorhynchus tshawytscha* eggs find out that best amount of hydrogen peroxide in comparison with formalin is 1000 mg/L for 15 min. With study on fungal diseases of fresh water fishes in southeast Asia from 1999 to 2004 one hundred and sixteen fungal isolate into 4 genera, namely *Saprolegnia*, *Achlya*, *Leptolegnia* and *Aphanomyces* were classified (Chukanhom, 2005). Hatai and Hoshiai (1992) found that signs of Saprolegniosis will subside when water temperature reaches above 18°C and this indicate that Saprolegnia species in lower temperature are able to become severe and epidemic. Depend on environmental differences and management conditions of farms, fungi have wide range infection (Willoughby, 1989). Surely this organisms in developing of fungal infections on eggs play an important role. It has been proven that Saprolegnia involved in environment with Fusarium has less growing in comparison with other environment, however, relation with saprolegnia and fusarium is not clear properly. In fact we can conclude, in this way that Saprolegniaceae are first fungi which exist in fungal infection of rainbow trout and adhere to died eggs. They grow and penetrate into cell wall and with reducing water flow and enzyme secretion which lead to death of eggs.

Conclusion

Existence of fungi in surrounding environment of fishes is considered as an obvious, but controlling these organisms in aquaculture and especially in hatcheries resulted to increase in egg yield and reducing external lesions in broodstock fishes. In present study we succeed to isolate 8 strains of fungus around eggs and outer surface of broodstocks bodies that among them, Saprolegnia has more importance, because it is from the most common fungal infections and need more attention from the point of health.

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