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Incidence of Histopathological and Molecular Identification of Some Causative Agent of Streptococcosis Isolated from Farmed Rainbow Trout (*Oncorhynchus mykiss*) in Mazandaran Province, Iran

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ABSTRACT: Over the past few years, the syndrome of streptococcosis has been associated with outbreaks in rainbow trout (*Oncorhynchus mykiss*) and caused important economic losses in the aquaculture industry in Iran especially in Mazandaran province. The main purpose of this work was molecular identification of some causative agents of streptococcosis in rainbow trout with emphasis histopathological and molecular identification of some causative agent of streptococcusis isolated from farmed rainbow trout (*Oncorhynchus mykiss*) in Mazandaran province on the north of Iran. The suspicious samples were also provided from a five contaminated Rainbow Trout farm in Mazandaran Province. Histopathological finding in kidney represent Depletation of hemopoitic tissue, acute necrotizing glomerulonephritis and Nectoric tubular epithelia, mononuclear cell infiltration, hemorrhage and tubular hyaline degenerations. Histopathological finding in spleen represent hemorrhagic foci and necrotic area, disarrangement cell structure, Spleen parenchyma from an infected fish showing multifocal hemosiderin deposition. In the current study, the host and pathogen bands with 725 bp and 340 bp lengths were observed in all the five samples. The aim of this study was to use molecular approaches to investigate the relative contributions of composition of rainbow trout infected by Streptococcusis emphasis whit histopathological lesion in kidney and spleen.

INTRODUCTION

One of the most important fish diseases which have caused outbreak in rainbow trout farm in Iran is streptococcusis (Sepahdari *et al.,* 2012). Infection by streptococcosis is one of the most significant bacterial disease in farmed salmonid fishes. The important pathogenic species of streptococcal infections are *Streptococcus parauberis*, Streptococcus iniae, *Streptococcus difficilis*, *Lactococcus garvieae*, *Lactococcus piscium*, *Vagococcus salmoninarum* and *Carnobacterium piscicola* (Bercovier *et al.*, 1997; Eldar *et al.*, 1997).

Infectious diseases of cultured fish are among the most considerable constraints on the expansion of aquaculture and the realization of its foil potential (Plumb, 1999; Woo and Bruno, 1999; Klesius *et al.*, 2000). Streptococcus iniae was first described as a pathogen of fish in 1994, when it was cultured from diseased tilapines *Oreochromis* spp (Eldar *et al.*, 1994, 1995). In addition to bacteria in the genus streptococcus, there are several other closely related groups of bacteria that can cause similar diseases such as: *Lactococcus garvieae* L. *piscium* and *Vagococcus salmoninarum* (Buller, 2004; Austin and Austin, 2007).

Molecular diagnostic techniques, such as PCR assays, are progressively used to detect and identify many different bacterial pathogens including the most significant fish pathogens such as Streptococcus species. Many of the PCR assays use the 16S rRNA gene as target molecule (Blanco *et al.,* 2002; Mata *et al.,* 2004).

In the present work we assessed the pathological outcomes of infections of rainbow trout by streptococcusis.

MATERIAL AND METHODS

The current study was conducted from August 2013 to August 2014 in Mazandaran, Iran. The suspicious samples were also provided from a contaminated Rainbow Trout farm in Mazandaran Province, Iran by the authorization of the Iranian Veterinary Organization.

A. Histopathological examination

The fish were quickly euthanized with (benzocaine, 60 mg/l) and measured for standard length and body weight. Then, the second gill from left side from individual fish of all groups was dissected and immediately fixed in 10% neutral buffered formalin for histopathological examination.

The tissue was later washed clean of formalin and passed through a series alcohol concentration to remove the water. The tissue were again passed through a chloroform / alcohol and pure alcohol therefore the gill sections, 5 µm thick, were cut with semi automate rotatory microtome RM2245 and then stained with hematoxylin and eosin and mounted on glass slide. Images were analyzed by light microscope and recorded with mioticcam 3000 photomicroscope.

B. DNA Extraction

Bacterial colonies were picked from culture and suspended in lysing buffer containing sucrose 320 mM, Tris 10 mM, $MgCl_2$ 5 mM, and 1% SDS. The tube was kept in a termoblock apparatus at 50°C for one hour. Then, bacterial DNA was extracted by phenol chloroform method and precipitated by absolute ethanol. DNA of fish tissue was also extracted according to the directions of manufacturer (Bioneer, South Korea).

C. Primer and Polymerase Chain Reaction

To prevent false-negative results in PCR method, two pairs of primers, based on 16S rRNA of S. iniae and 18S rRNA of *Oncorhynchus mykiss* (1), with the following sequences were applied in the multiplex PCR:

16S rRNA:
STRinF 5'-GGTAAGCCGTATCGGAAGGT-3'
STRinR 5'-CCTAGC-3'TCCTTGTCAATGGAG
18S rRNA:
Onmy F 5'-CTGTGGCAATTCTAGAGC-3'
Onmy R 5'-CGTCCCTCTTAATCATGG-3'

The final volume was adjusted to 23 µl with 10 µl 2X Ampliqon 2 Master Mix, 1 µl *S. iniae* Primer R/F, 2 µl (40 pmol each) *O. mykiss* Primer R/F, 3 µl (1 µgr) template DNA, 7 µl DW.

PCR final product using S. iniae 16S rRNA and 18S rRNA primers were 340 bp, which had significant difference. PCR was amplified under the following conditions: early denaturation at 94°C for 10 minutes in 30 cycles containing denaturation for 45 seconds, annealing at 53°C for 45 minutes, and extension at 72°C for one minute, and finally the reaction was completed at 72°C for five minutes.

D. Electrophoresis

After completing the reaction, 10 µl of PCR product was electrophoresed on 1.5% gel agarose and illustrated by 250 nm wavelengths UV in UV DOC apparatus. The samples were loaded beside a 100bp marker.

After adjusting the system, at least five samples, which their contamination had been approved by histopathological methods, were tested by the MultiPlex PCR method.

RESULTS

Histopathological examination in kidney was showed Depletation of hemopoitic tissue and acute necrotizing glomerulonephritis and enlargement of glomeruli Kidney (Fig. 1) and histopathological examination in Spleen of the rainbow trout infected with Streptococcus was showed Hemorrhagic foci, necrotic area and disarrangement cell structure and Spleen parenchyma from an infected fish and showing multifocal hemosiderin deposition (Fig. 2). Considering the aforementioned MultiPlex PCR method, proliferation DNA segment of the host implied proper amplification and detection; in such a way that, observing two bands of the host and the pathogen indicated proper conduction of PCR and contamination of the suspected samples; the segments with 752 bp and 340 bp belonged to the host and the pathogen, respectively (Fig. 1).

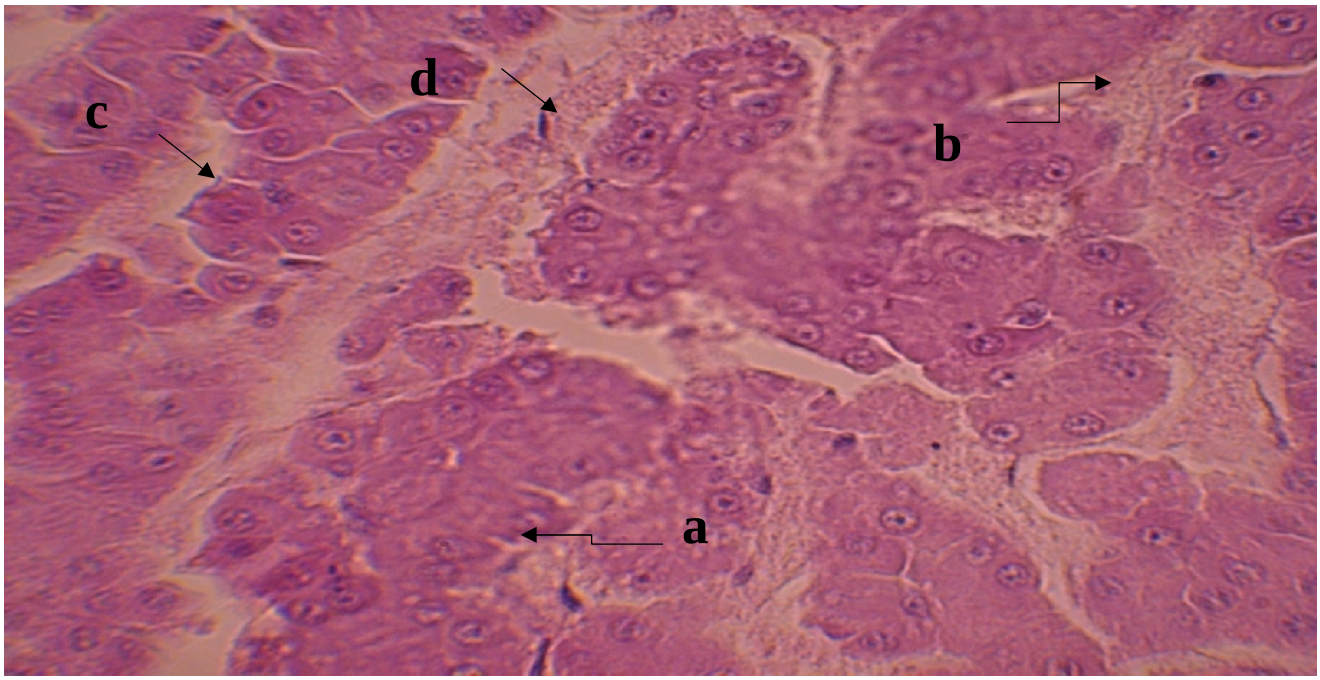

Fig. 1. Showing kidney of contaminated fish that represents Depletation of hemopoitic tissue and acute necrotizing glomerulonephritis (a) Kidney of the rainbow trout and Nectoric tubular epithelia. And showing mononuclear cell infiltration (b), hemorrhage (c), tubular hyaline degenerations (d). in kidney (H&Ex100).

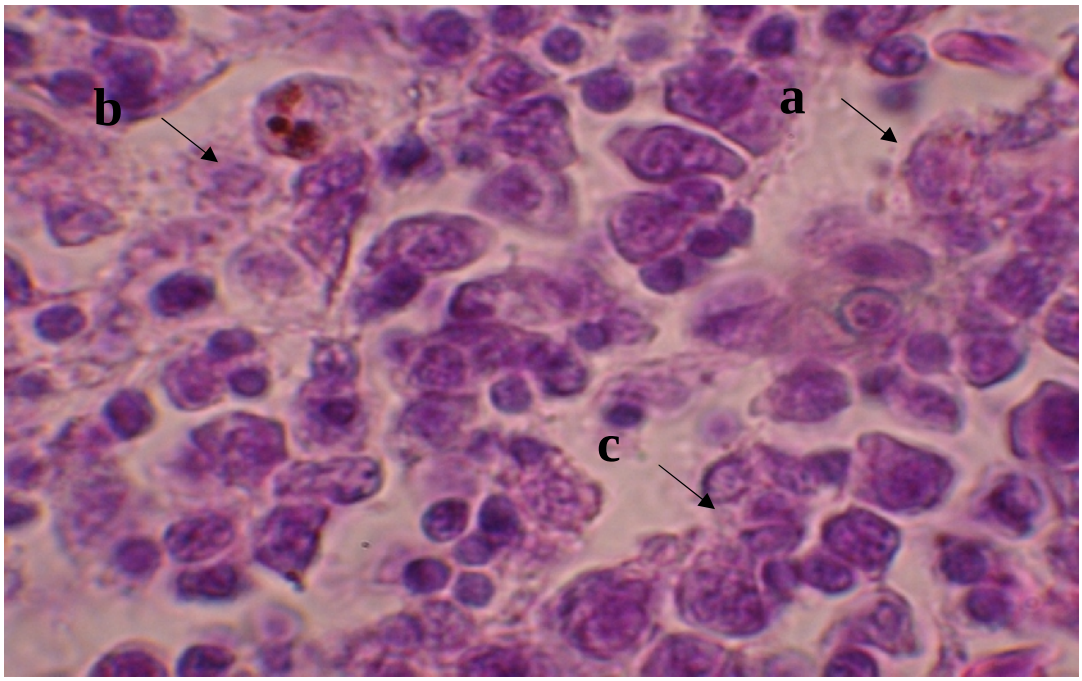

Fig. 2. Spleen of the rainbow trout infected with. (a) *Streptococcus* Hemorrhagic foci and necrotic area, (b) disarrangement cell structure (c) Spleen parenchyma from an infected fish showing multifocal hemosiderin deposition (H&Ex100).

Observing only the host related band in the PCR product implied proper PCR conditions and showed that the sample was not infected with *S. iniae*; this result was definite, without common test errors (Haghighi *et al.*, 2015).

Observing two pairs of *S. iniae* and the fish designed primers simultaneously with the DNA extracted from the contaminated fish tissue as a template in MultiPlex PCR implied that PCR was conducted under improper conditions and common test errors. In the current study, the host and pathogen bands with 725 bp and 340 bp lengths were observed in all the five samples.

DISCUSSION

The aim of this study was to use molecular approaches to investigate the relative contributions of composition of rainbow trout infected by Streptococcusis emphasis whit histopathological lesion in kidney and spleen. The most consistent clinical sign of infection with Streptococcus disease in trout is the development of exophthalmus and other serious eye lesions. Similar eye lesions are described in outbreaks of streptococcal infection in cultured turbot (Domenech, Fernandez-Garayzabal, Pascual, Garcia, Cutuli, Moreno, Collins and Dominguez 1996) and in tilapia and channel catfish (Chang and Plumb, 1996). Using internal control samples to diagnose some human pathogen microorganisms is practically exploited; in such a way that, using these internal control samples to increase the accuracy of diagnosing fish farming diseases still paves the early steps and has to be developed.

Over the past few years, streptococcosis has been the most rampant infectious bacterial disease in cold freshwater fish (rainbow trout) farms in Iran. This is a serious problem which causes economic losses every year in aquaculture industry, as it was reported by Akhlaghi and Keshavarzi, 2002; Soltani *et al.*, 2005, 2008; Saeedi *et al.*, 2009; Pourgholam *et al.*, 2010, in press) in some provinces of Iran.

Finally, in other researches two species *Streptococcus iniae* and *Lactococcus garvieae* were isolated from rainbow trout farms of Fars, Mazandaran, Gilan and Tehran provinces of Iran (Soltani *et al.*, 2005; Soltani *et al.*, 2008). Streptococcosis in acute stage of disease can cause huge mortality with no clinical sign. In incubation period of disease fish showed no clinical sign too. This fish may be marketed due to lack of awareness of fish farmers about disease. However, in the present study, approximately 35% of specimens were infected to Streptococcus species that in all infected cases pathological symptom been felt in this study is well illustrated histopathological study prepare the way for further studies.

In the spleen of infected tilapia, we found haemorrhages in intercellular spaces together with multiple foci of melanin deposits and melano-macrophage centers (MMC). These MMCs are macrophage aggregates containing pigments such as hemosiderin, melanin and lipofuscin (Marzok *et al.*, 2009). Many studies have demonstrated that an increased size and number of MMCs were more apparent in livers and spleens of fish infected with bacteria (Alagappan *et al.* 2009; Miyazaki *et al.*, 2001). Findings similar to sluicy observation, including pericarditis, infiltration of macrophages and lymphocytes into internal organs, hyaline deposition in tubular cells in kidney, and meningitis were documented. In addition, our current results were in agreement with many recently published reports which have shown that naturally infected fish with streptococcal disease showed a variety of pathological conditions, including congestion of internal organs, particularly in the liver, spleen and kidney and brain (Bowater *et al.*, 2012; Chen *et al.*, 2011; Chen *et al.*, 2003).

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