Short Communication

Experimental study to evaluate the pathogenicity of *Streptococcus iniae* in Guppy (*Poecilia reticulata*)

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Abstract: *Streptococcus iniae* has emerged as an important fish pathogen over the last decade in farmed rainbow trout in Iran. The main objective of this study was to evaluate the pathogenicity of *S. iniae* in *Poecilia reticulata*. A total of 60 apparently healthy *P. reticulata* were obtained from ornamental fish pet store and injected intraperitoneally with 1.5×10^6 cfu of bacteria. For 14 days after challenge, the rate of mortality and clinical signs were recorded. The first clinical signs was observed in challenged fish 48 hrs after injection of *S. iniae* and first mortality was observed 72 hrs after injection. No significant differences in mortality and clinical signs between both sexes were observed. *Streptococcus iniae* was collected from internal organs of fishes challenged, and was confirmed using the conventional biochemical tests and PCR. It is concluded that, *P. reticulata* is susceptible to streptococcosis and can play an important role in transmission of the disease to other ornamental fish species and also cultured fish.

Introduction

Streptococcosis/lactococcosis is one of the most important bacterial diseases in farmed rainbow trout in Iran. This disease was reported in wild and farmed fishes in fresh and salt water, and also in ornamental fishes (Austin and Austin, 2007). The main pathogenic species that have been associated with this disease include *Streptococcus iniae*, *S. agalactiae*, *S. parauberis*, *S. dysgalactiae*, *S. faecium*, *S. milleri*, *S. uberis*, *S. ictaluri*, *S. phocae*, *S. faecalis*, *Lactococcus garvieae*, *L. piscium*, *Carnobacterium piscicola* and *Vagococcus salmoninarum* (Shoemaker et al., 2000; Russo et al., 2006). *Streptococcus iniae* and *L. garvieae* are the major pathogens of streptococcosis and lactococcosis in the cultured farms in Iran (Akhlaghi and Keshavarzi, 2002; Soltani et al., 2005). The occurrence of this disease

in the cultured rainbow trout in Iran was reported by Akhlaghi and Keshavarzi (2002), Soltani et al. (2005), Soltani et al. (2008), Sharifiyazdi et al. (2010), Pourgholam et al. (2011), Fadaeifard et al. (2012) and Rahimi Kia and Mehrabi (2013).

Streptococcus iniae is the etiological agent of a hemorrhagic septicemia, characterized by anorexia, uni or bilateral exophthalmia, blackening of the skin, abdominal distension and hemorrhages in the internal and external organs (Agnewa and Barnes, 2007). Also, *S. Iniae* described as an important zoonotic bacterial disease causing cellulites and endocarditis in humans (Agnewa and Barnes, 2007). *Streptococcus iniae* was isolated from different ornamental fishes; including Red-tail black shark (*Epalzeorhynchos bicolor*), Rainbow shark (*E. frenatum*), *Erythrurus erythrurus*, zebra danio (*Danio rerio*), Pearl danio (*Danio albolineatus*),

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Clown loaches (*Chromobotia macracanthus*), *Botia macracanthus*, *Barbus conchonius*, *Hyphessobrycon* sp., and African cichlids (Russo et al., 2005; Raissy et al., 2012; Ferguson et al., 1994).

In the last 10 years, the ornamental fish industry has developed in Iran (Meshgi et al., 2006). There are 150 species of aquarium fishes, and about 40 of them are bred and raised throughout the country (Meshgi et al., 2006). The Guppy (*Poecilia reticulata*), is a small member of the live-bearing Poeciliidae family (Breden et al., 1987). Despite the importance of *S. iniae* in streptococciosis outbreaks in Iran, there are few researches about the agent of disease in ornamental fish. Hence, the aim of this study is to evaluate the pathogenicity of *S. iniae* in *P. reticulata*.

Materials and methods

Fish: A total of sixty apparently healthy *P. reticulata* (each sex: 30 specimens) were obtained from an ornamental fish store (Sari, Mazandaran Province, north of Iran) in April 2013. The average weight of females and males were 0.8-1 (0.8 ± 0.2) g and 0.4-0.7 g (0.4 ± 0.3), respectively. Also, the average length of female and males were 3-6 (4 ± 2) cm and 2.5-4 (2.5 ± 1.5) cm, respectively. Fish were transferred to fish diseases laboratory at the Sari University of Agricultural Sciences and Natural Resources, in aerated tank using a portable air pump and introduced to a 200 L glass aquarium. Fish were divided into control and two treatments (including male and female groups) each includes 20 specimens. The water temperature, DO and pH of aquariums were 28 ± 1°C, 4.9 ± 0.2 mg l^{-1} and 7.6 ± 0.2, respectively. *Poecilia reticulata* were kept in these aquaria for one week for adaption period and fed by similar diet (Taksiran Company, Iran).

Bacterial strains and Challenging: *Streptococcus iniae* was isolated from kidney of moribund *P. reticulata* showing the signs of streptococosis. The isolated bacteria were confirmed using the conventional biochemical tests and molecular methods (PCR). In preparation a stock culture, the bacteria were purified in TSA at 30°C for 20 hrs. A

few of the resultant purified colonies were grown for 20 hrs at 30°C in three 150 ml brain heart infusion (BHI) broth. The isolate was adjusted to McFarland turbidity standard No. 3, which was equivalent to 1.5×10^6 cfu/ml. All the fish in two groups were intraperitoneally challenged with 0.1 ml of *S. iniae* (1.5×10^6 cfu/ml). Fourteen days after challenge, the mortality rate and clinical signs were recorded. Data were analyzed using SPSS (Version no. 18) and significant differences between to both sex were determined by a one-way analysis of variance (ANOVA).

Isolation of bacterium and bacteriological examination: Sampling was performed from kidney, brain and liver of challenged fish that showed clinical signs in the aseptic condition, then directly streaked by sterile swabs on blood agar supplemented with 1.5% NaCl and Tiosulfate Citrate Bile Salt agar (TCBS). Plates were incubated at 30°C for 48 hrs. Then, the macroscopic and microscopic observation of the colonies, single colonies with pure culture growth were subcultured onto BHI and identified using the conventional biochemical tests.

Identification of the isolated bacteria by PCR: DNA was extracted using a DNA isolation kit (MBST, Iran) according to the manufacturer's instructions. Two pairs of primers, including F (5′-CTT ACC TTA GCC CCA GTC TAA CGA C-3′) and R (5′-GTC GTA ACA AGG TAA GCC GTA TCG –3) were used to identify *S. iniae* (Soltani et al., 2005). The primers were synthesized by CinnaGen Company (Tehran, Iran). The expected 513 bp PCR amplification product confirmed the biochemical identification. The PCR was performed in a total reaction volume of 50 µl, containing 500 mM KCL, 100 mM Tris-HCl (pH 9.0), 60 mM MgCl, 200 µM dNTPs, 1 µl of each primer and 0.2 U Taq DNA polymerase per 50 µl reacti, 1 µl of template DNA and 40 µl of sterile distilled. Distilled water was used as a negative control in each PCR reaction. The reaction was repeated for 37 cycles under the following conditions: 3 min at 94°C (1 cycle), 1 min at 94°C, 1 min at 45°C, 1.5 min at 72°C (35 cycle)

Figure 1. Blackening of skin, abdominal distension, hemoraghes and anal prolaps in the female *P. reticulata* challenged by *S. iniae*.

and finally PCR were completed with the final extension step at 72°C for 10 min. PCR products were separated on 2% agarose gel in 0.5× Tris–borate–ethylene diamine tetra acetic acid (EDTA) buffer and visualized using ethidium bromide and an UV illuminator.

Results

No mortality and clinical signs were observed in the control group. The first signs of disease were observed in challenged specimens 48 hrs and first mortality 72 hrs after injection. The first clinical signs were the lethargy, anorexia, erratic swimming and bottom siting. Also, blackening of the skin, abdominal distension, hemorrhoids and anal prolapse (Fig. 1), uni and bilateral exophthalmia sometimes with hemorrhage (Fig. 2), accumulation of blood fluids in the abdominal cavity, hemorrhages in the external surface (Fig. 3) and in internal organs, including the liver, kidney and intestine were observed in some moribund fish. No significant differences in mortality and clinical signs between sexes were observed. The isolated bacteria were confirmed using the conventional biochemical tests and PCR (Austin and Austin, 2007).

Discussion

Streptococcus iniae is a major fish pathogen causing streptococcosis and zoonotic bacterial disease. Streptococcosis in the cultured fish is considered as a major problem in Iran causing a significant economic losses in the aquaculture industry, especially in the rainbow trout. The occurrence of streptococcosis in the rainbow trout farms was reported in different parts of Iran (Soltani et al., 2005). In addition, this disease was reported in

Figure 2. Uni and bilateral exophthalmia in the male *P. reticulata* challenged by *S. iniae*.

Figure 3. Hemorrhages in the external surface in the female *P. reticulate* challenged by *S. iniae*.

different ornamental fish in some countries (Russo et al., 2005; Raissy et al., 2012; Ferguson et al., 1994), whereas, there are few reports of this disease in the ornamental fish industry in Iran (Raissy et al., 2012). In this present study, the first signs of disease observed in the challenged fish two days after injection of *S. inane* and mortality observed in third day. Raissy et al. (2012) evaluated the pathogenicity of *S. iniae* in Silver Shark and Rainbow Shark (*E. frenatum*). The clinical signs in these species were similar to the results of present study with differences in outbreak time of clinical signs and mortality (Raissy et al., 2012) that could be a species specific or different strain of *S. iniae*. Furthermore, the observed clinical signs of this study are similar to those of reported in rainbow trout and other ornamental fish (Austin and Austin, 2007; Ferguson et al., 1994).

In conclusion, *P. reticulata* is susceptible to streptococcosis and can play as carrier. This species is able to transmit of this bacteria to other ornamental fish species and also cultured fish. It is recommended an extensive studies to evaluate of pathogenicity of *S. iniae* in other ornamental fish.

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