

## Survey on viability of *Streptococcus iniae* in frozen rainbow trout (*Oncorhynchus mykiss*) following experimental infection

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### ABSTRACT

*Streptococcus iniae* is one of the most important agents that causes high mortality and losses in rainbow trout farms in Iran and the world. These bacteria can affect the consumers and people who deal with aquaculture via affected fish. So, viability of this bacterium in frozen fish and fish product can be useful for health care of consumers. A total of 90 rainbow trout, with average weight of  $50 \pm 3$  g, were supplied from a fish farm and were transferred to six 200-liter tanks (15 fish per tank). The tanks were divided into two groups as control and treatment. The treatment group fish were experimentally challenged to *Streptococcus iniae* at a dose of  $3.6 \times 10^5$  cell/fish via intraperitoneal injection. Control group fish just received 0.1 ml of normal saline (0.9% NaCl). In the treatment group after 120 hours 91% of the fish were died and then mortality remained constant up to 14 days post challenge. Moribund and dead fish of the treatment group and fish of the control group were frozen and then kidney, liver and brain of fresh dead and frozen fish were used for bacterial culture after 8, 14, 20, 26, 30, 34, 36 and 38 months post freezing, to investigate the viability of these bacteria in different organs at freezing temperature ( $-20^\circ\text{C}$ ). *Streptococcus iniae* were re-isolated from all of fresh and frozen tissue specimens within 38 months after freezing. All bacterium species confirmed as *Streptococcus iniae* using polymerase chain reaction (PCR) method. According to this study this bacterium as a zoonosis can be survived at least 38 months in freezing temperature (at  $-20^\circ\text{C}$ ) in fish products, so the potential pathogenicity of this bacterium should be concerned in affected areas as well as imported frozen fish.

### 1. Introduction

Iran, with a remarkable increase in production of rainbow trout during the past few years, has been ranked as the third biggest producer of farming trout in the world with producing 62.6 thousand tons of this fish in 2010. (Adeli and Baghaei, 2013). In aquaculture because of limitation in fresh water sources, farmers increase fish density in ponds. Elevation of fish density leads to high

susceptibility of fish and outbreak of various infectious diseases in reared fish. Some of these diseases can affect and cause disease in humans too. *Streptococcus* agents are one of these zoonoses. This disease was first reported in 1957 in rainbow trout from Japan (Hoshina et al., 1958), and thereafter, was rapidly reported in different fish species including turbot, trout, salmon, bass, tilapia, yellow tail and etc from different countries such as Italy, Japan, USA, Bahrain, Saudi Arabia, Turkey, Spain, Australia,

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Canada, Kuwait and Iran (Inglis *et al.*, 1993; Kusuda *et al.*, 1976; Kusuda *et al.*, 1978; Edlar *et al.*, 1999; Al-Habri, 1994; McNulty *et al.*, 2003; Russo *et al.*, 2006; Cheng *et al.*, 2010; Ynag and Li, 2010; Austain and Austin, 2007; Nieto *et al.*, 1995; Yuasa *et al.*, 1999; Soltani *et al.*, 2008; Azad *et al.*, 2011).

This disease can cause more than 70% mortality during the warm months in fish farms (Bromage *et al.*, 1999). Different species of *Streptococcus* cause disease in the fish farms, of which, the most prevalence belonged to *Streptococcus iniae*. This species can cause disease in different reared and wild fish, and was reported at least from 27 fish species (Agnew and Barnes, 2007).

In Iran streptococcosis was first reported in 2001 in rainbow trout farms from Fars province and thereafter was rapidly reported from the other provinces of Iran too (Akhlaghi and Keshavarzi, 2002; Soltani *et al.*, 2005; Soltani *et al.*, 2008). It is now one of the most common diseases in the coldwater farms of Iran that causes huge economical loses in the trout farms.

To date two species including *Streptococcus iniae* and *Lactococcus garvieae* have only been isolated from the rainbow trout farms of Iran as the agents of streptococcosis symptoms. They have not been reported from other fish species in Iran (Soltani *et al.*, 2005; Soltani *et al.*, 2008). On the other hand *Streptococcus iniae* can cause disease in human too. There are several reports about the transmission of this disease from infected fish to human, where the main clinical signs in patients were arthritis, endocarditic, meningitis and spinal osteomalacia (Weinstein *et al.*, 1997; Goh *et al.*, 1998; Lau *et al.*, 2003; Lau *et al.*, 2006). Therefore, this disease should be more concerned in fish industry and human health. Trouts are marketed fresh or frozen, while its trade is mostly taken in frozen form. Freezing less and more can destroy parasites but bacteria have more freezing tolerances. Thus, *streptococcus* infected fish can cause problems in consumers and the people who are dealing with. As streptococcosis is a zoonotic disease, information about its way of transmission, resistance to freezing and survival rate in the aquatic products can be useful for a better consumers and public health.

## 2. Material and Methods

### 2.1. Fish rearing conditions

In the autumn 2010, a total number of 90 rainbow trout with average weight ( $\pm$  SD) of  $50 \pm 3$  g were supplied from a fish farm and transferred to six 200- liter tanks (15 fish per tank) with water flow rate of 8 L/min. The fish were divided into two groups including three tanks as control and three tanks as experimental group. Fish were acclimatized for two weeks. During the acclimation period, the fish were daily fed by commercial food (BioMar Co.) at rate of 3% of biomass. During the rearing period, water temperature, pH and dissolved oxygen maintained at  $16 \pm 3^\circ\text{C}$ ,  $7.1 \pm 0.2$  and  $7.5 \pm 0.4$  ppm, respectively.

### 2.2. Bacteria culture and the model of challenging

*Streptococcus iniae* that had been isolated from natural rainbow trout infected farms was supplied from bacterial collection of Microbiology section. This bacterium was cultured on nutrient agar (Merck Co., Germany) plus 5% defibrinated ship blood for 24 hours at  $28^\circ\text{C}$ . The colonies thereafter were collected and a homogenous suspension was prepared using normal saline (NaCl 0.9%). Initial stocks including  $10^8$  cell/ml of bacterial suspension prepared by spectrophotometer (640 nm and OD =1) and  $3.6 \times 10^6$  cells/ml viable bacteria were prepared using serial dilution and determined according to Spread Plate method (based on CFUs). Afterward, all fish of control and treatment groups were anesthetized by clove oil (100 ppm), then fish of treatment group received 0.1 ml of the mentioned bacterial suspension (containing  $3.6 \times 10^6$  cells/ml) via intraperitoneal injection. The control fish were intraperitoneally injected with 0.1 ml of sterile normal saline (0.9 % of NaCl) and were kept in an isolated place. After injection, the fish were monitored daily for 14 days post challenge. The fish behavior and clinical signs were recorded and lethargic and dead fish were removed and after clinical signs recording and sampling were frozen at  $-20^\circ\text{C}$ .

### 2.3. Re-isolation of bacteria

Kidney, liver and brain of fresh dead and frozen fish were used for bacterial culture after 8, 14, 20, 26, 30, 34, 36 and 38 months, to investigate the viability of the bacteria in different organs of the frozen fish. To this, for each stage of experiment, 5 frozen fish specimens were investigated. Frozen fish were kept at refrigerator (4-5°C) for 24 h for defrosting. Then, their abdomen was opened and the kidney, liver and brain samples were used for bacterial culture on nutrient blood agar medium. After growth and colony formation on bacterial culture plate, pure bacteria isolated. Finally, molecular identification method (PCR) was used for confirmation of bacteria. Biospin Bacteria Genomic DNA Extraction Kit (Bioflux, Japan) was used to extract DNA from isolated gram positive and catalase negative cocci. Agarose gel (1%) electrophoresis was applied to determine the DNA quality and quantity. The method recommended by Soltani *et al.* (2005) was followed to identify *Streptococcus iniae* isolates.

## 3. Results

### 3.1. Mortality and clinical signs of challenged fish

Mortality was started 24 hours after injection of  $3.6 \times 10^5$  bacterial cells per fish in treatment group; and after 120 h, 91 % of the fish were dead and then mortality remained

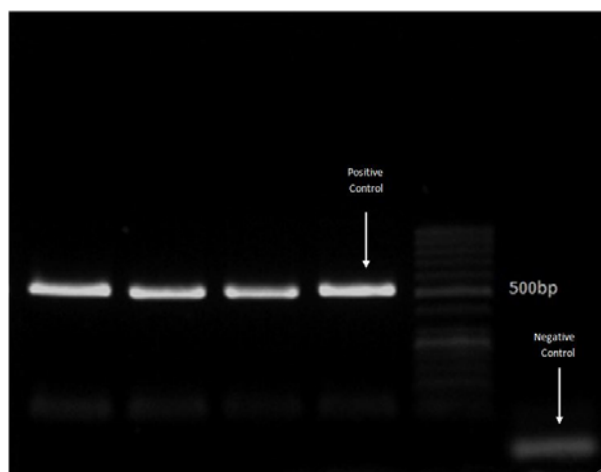
constant up to 14 days post challenge, whereas, there was no mortality in the control group. The fish that died within 24 h after injection had no specific clinical symptoms. After 24 hours post challenge clinical signs were observed, which mainly were exophthalmia, up and down swimming, lethargy, skin darkness, cataract and petechia in eyes, hemorrhage on body surface specially in fin bases, congestion in intestines and accumulation of bloody fluid in abdomen cavity. In control group fish no mortality were observed during 14 days post challenge.

### 3.2. Culture and re-isolation of bacteria from the dead fish

According to the result, a gram positive, catalase negative and beta hemolytic cocci bacterium was re-isolated from all fresh fish of treatment group. This bacterium was identified as *Streptococcus iniae* using polymerase chain reaction (PCR) method. The results of survival and viability of this bacterium in frozen fish are shown in table1. In this study, *Streptococcus iniae* was re-isolated on nutrient blood agar, from 100 % of kidney, liver and brain specimens of the frozen treatment fish group at -20°C during 38 months (within 8, 14, 20, 26, 30, 34, 36 and 38 months in freezing temperatures). *Streptococcus iniae* was confirmed in all specimens using polymerase chain reaction (PCR) method. In control group, no *streptococcus iniae* was re-isolated from both fresh and frozen fish.

**Table 1.** The percentage of survival rate of *Streptococcus iniae* in different tissues of treatment fish (challenged with  $3.6 \times 10^5$  bacteria) and control fish (challenged with normal saline including any bacteria) during freezing at -20 °C

	Organ sampled	Sample size for each stage	Percentage of re-isolated <i>Streptococcus iniae</i> from the tissues kept at -20°C after experiment examination							
			8 months	14 months	20 months	26 months	30 months	34 months	36 months	38 months
Treatment group	Liver	5	100%	100%	100%	100%	100%	100%	100%	100%
	Kidney	5	100%	100%	100%	100%	100%	100%	100%	100%
	Brain	5	100%	100%	100%	100%	100%	100%	100%	100%
Control group	Liver	5	0 %	0 %	0 %	0 %	0 %	0 %	0 %	0 %
	Kidney	5	0 %	0 %	0 %	0 %	0 %	0 %	0 %	0 %
	Brain	5	0 %	0 %	0 %	0 %	0 %	0 %	0 %	0 %



**Figure 1.** Gram positive cocci bacteria re-isolated from kidney, liver and brain of treatment group fish was identified as *Streptococcus iniae* 38 months after freezing at  $-20^{\circ}\text{C}$  by PCR technique. Positive control: verified *Streptococcus iniae*, negative control: adjuvant fluid without any DNA product.



**Figure 2:** Gram positive, beta hemolytic cocci bacteria was re-cultured on nutrient blood agar from brain, kidney and liver of treatment fish group after freezing at  $-20^{\circ}\text{C}$

#### 4. Discussion

For the first time, *Streptococcus* bacteria was reported from fish farms by Akhlaghi and Keshavarzi (2002) from Fars province of Iran. Biochemical examinations showed that the isolated *Streptococcus* was 95.65 and 82.6% similar to *Streptococcus iniae* and *Lactococcus garvieae*, respectively. 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 (Soltaniet 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. Most reared fish in Iran are marketed fresh and kept frozen until consumption. In this study, *Streptococcus iniae* was isolated from 100% of kidney, liver and brain tissues, 38 months after freezing at  $-20^{\circ}\text{C}$ . Therefore, this species is resistance to freezing, and if hygienic principles are not considered, the consumer and those who are dealing with these fish may face problems. In previous study Evans et al. (2004) showed that *Streptococcus agalactiae* can be survive for 6 months at  $-20^{\circ}\text{C}$  and  $-70^{\circ}\text{C}$  in frozen fish. There aren't a lot of studies about survival rate of *Streptococcus iniae* in long term freezing period. Zoonotic nature of this disease was first considered in 1994-1995, when 9 persons from Toronto were

infected with this bacterium. All patients were Asian and involved with fresh and unprocessed fish. Also, 8 of them had cellulitis in their hands and one person showed endocarditis, meningitis and arteritis symptoms. According to the reports of microbiology laboratory CDD, due to the infection with this bacterium, two persons showed clinical signs of cellulitis in Texas in 1991 and one cook was sick with this disease in Ottawa in 1994 (Weinstein et al., 1997). Goh et al. (1998) also reported two patients due to this disease in Vancouver, Canada. Lau et al. (2003) reported two patients infected with this bacterium, which one of them had cellulitis in hand and the other one had spinal osteomyelitis. Koh et al. (2004) reported three patients with spinal infection that *Streptococcus iniae* identified as main agent of this disease; all of these three patients were Chinese. Lau et al. (2006) reported two patients infected with *Streptococcus iniae* in Hong Kong. They were also Chinese and over 60 years old. According to Lau et al. (2006), isolated *Streptococcus iniae* isolated from Asia had more hemolytic characteristics on nutrient blood agar and produced more mucoid colonies in compared to the pathogenic strains isolated *Streptococcus iniae* from North America. Likewise, these researchers suggested that Chinese and older are more susceptible to disease. However, this claim needs more studies to be proven. At least 25 human case reports have been available until 2007 (Agnew and Barnes, 2007). Although these rates appear to be low, but it should be noted that many streptococcosis are not recorded and reported, particularly in developing countries. So it can be said that the real outbreak of this disease in human is higher than the reported cases. On the other hand, considering the high resistance of the bacterium under maintenance procedures of fish and aquatic products, human infestation with *Streptococcus iniae* should be more concerned.

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