Epizootic outbreaks of lactococcosis caused by *Lactococcus garvieae* in farmed rainbow trout (*Oncorhynchus mykiss*) in Iran

M. Soltani[1*], Gh. Nikbakht[2], H.A.E. Mousavi[1] and N. Ahmadzadeh[3]

[1] *Department of Aquatic Animal Health, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran;* [2] *Department of Microbiology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran;* [3]*Central Research Laboratory, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.*

Abstract

Lactococcus garveiae outbreaks in farmed rainbow trout are described from different parts of Iran. The disease outbreaks were associated with water quality parameters particularly high water temperature (>14°C) and poor health management. The peak of outbreaks occurred during late spring till late summer with mortalities varied from 10-50%. The disease was diagnosed in fish of mainly above 50 g in seven provinces of Iran located at the northwest, northeast and southwest. The phenotypic and molecular studies of the recovered isolates of bacteria showed that they belonged to both *L. garvieae* and *Streptococcus iniae* but isolation of *L. garvieae* was more frequent than *S. iniae*.

Introduction

Lactococcus garvieae, previously described as *Streptococcus garvieae* and *S. iniae* are two of the most important Gram positive pathogenic cocci of several commercially important fish species including yellowtail, rainbow trout, tilapia, sea bream and sea bass (Ceschia et al., 1992; Carson et al., 1993; Toranzo et al., 1994; Eldar et al., 1999; Kusuda & Salati, 1999; Pereira et al., 2004; Soltani, 2005; Vendrell et al., 2006; Savvidis et al., 2007). In the affected regions the diseases caused by these bacteria are the major threat for rainbow trout culture, in particular during the warm seasons of the year. The losses produced can exceed 50-80% of the total production (Vendrell et al., 2006). *L. garvieae* has been also reported as the cause of mortality in gaint freshwater prawn *(Macrobrachium rosenbergii*) (Chen et al., 2001). In previous study by Soltani et al. (2005) outbreaks by *S. iniae* was reported from some farmed trout in south west of Iran. This manuscript describes the outbreaks of *L. garvieae* in several provinces of Iran between 2005 and 2007.

Materials and methods

Affected fish samples were collected from 10 trout farms located in seven provinces of the country including Mazandran, Tehran, Markazi, Kermansha, Lorstan, Fars and Charmohal-va-Bakhteyari (Figure 1). Totally 150 samples (15 samples from each trout farm) were used during spring till summer 2005-2007. The weight of the affected fish ranged from 30 to 400 g.

* Corresponding author's E-mail: msoltani@ut.ac.ir

Gross pathology was assessed through macroscopic evaluation of external appearance and lesions of the internal organs. Bacterial isolation was carried out by spreading samples from internal organs of spleen and kidney and in some cases from brain and endophthalmic fluid on to tryptic soy agar supplemented with 5% defibrinated sheep blood. The agar plates were incubated at 20-25°C for 48-72 hours. Isolated bacteria were identified according to phenotypic features (Gram stain, O/F, utilization of sugars, oxidase, catalase, VP, MR etc). The confirmation of thses bacterial isolates was obtained by PCR assay described by Soltani et al. (2005) using a set of primers, primer F (5'- GAG TGA AGA AGG TTT TCG GAT CG-3') and primer R (5'- TCC ATT GTA GCA CGA GTG TAG CC-3') designed from the 16S rRNA sequence (GenBank accession no. X54262. These primers amplify a fragment of 835 bp. The strains of *S. iniae* (FD strain (Soltani et al., 2005) and *L. garvieae* (TKS KG+, Japanese strain) were used as the positive control strains.

Results and discussion

Affected fish showed the typical signs of the disease, with a rapid anorexia and uni-or bilateral exophthalmia. In some cases, we also observed hemorrhagic signs and the loss of eyes. Internally, the diseased fish revealed a bacterial septicemia showing hemorrhagic fluid in the abdominal cavity and lumen of intestine, pale liver, splenomegaly and intestinal hyperemia. Gram stain preparation from imprinted smears of spleen, kidney and eye fluid showed huge numbers of Gram-positive cocci.

Mortality was concurrent with the water temperature with the highest mortality of up to 50% revealed during summer time. Water temperature during summer season increased up to 18-19°C in some of the diseased farms. When water temperature decreased during late summer the outbreaks were significantly reduced. However, the outbreaks continued during autumn and winter but with a sporadic mortality. The disease outbreak has economically a significant impact due to high mortalities related to fish size because the affected fish are mainly market sizes (above 250 g) (Vendrell et al., 2006).

Pure cultures of pale grey-whitish small colonies were observed after 2-3 days post-incubation. Totally 82% (123 samples) were positive in plate culture. All isolates were Gram-positive cocci, non-motile, oxidase and catalase negative (Table 1). Isolates were tolerant 6% NaCl, pH 9.5 and temperature 15-40°C (Table 1). Other phenotypic features of these isolated bacteria are listed in Table 1. According to these phenotypic characteristics 75 (61%) of these isolates were identified as *L. garviea*e. The rest recovered isolates (48 samples, 39%) were identified as *S. iniae* (Table 1) (Toranzo et al., 1994; Muzquiz et al., 1999). The confirmation of the recovered *L. garviea*e like-bacteria isolates was performed by PCR assays resulting in the amplification of a band of 835 bp which is expected for *L. garvieae* (Figure 2) (Soltani et al., 2005). The PCR analysis of *S. iniae* like-isolates showed that most of these isolates belonged to *S. iniae* (Soltani et al., 2005).

It is notable to say that the water source of diseased fish farms is mainly river. The river

Character	*Lactococcus garvieae*	*Streptococcus iniae*
Gram stain	Cocci (cocci)	Cocci (cocci)
Morphology	+ (+)	+ (+)
Hemolysis	a/ß(a/ß)	a/ß (a /ß)
Catalase	-(-)	-(-)
Oxidase	- (-)	-(-)
Motility	- (-)	-(-)
O/F	+/+(+/+)	-(F)
Citrate	+(-)	-(?)
NO3	+/-(-)	-(?)
Indole	-(?)	-(-)
MR/VP	+/-(+ /-)	+/-(+/-)
Glucose	+(+)	+(+)
Lactose	+/-(+)	-(-)
Maltose	+(+)	V(?)
Manitol	(V)	+(+)
Sucrose	V(?)	+(+)
Inositol	-(-)	-(-)
Arabinose	-(-)	-(-)
Xylose	-(-)	-(-)
Lysine	-(-)	-(-)
Ornithine	-(-)	+(?)
Arginine	+(+)	+(+)
Esculin	+(+)	+(+)
Urea	-(?)	-(?)
Gelatin	+(?)	-(-)
H2S	-(-)	-(-)
Growth at:		
NaCl (0-6%)	+(+)	+(+)
pH (5-9.5)	+(+)	+(+)
Temperature (15-40°C)	+(+)	+(+)
Ampicilline (10µg)	S(?)	S(S)
Erythromycine (15µg)	V(S)	S(V)
Oxytetracycline (30µg)	V(S)	S(V)
Enrofloxacin (10µg)	S(?)	S(?)

Table 1. Phenotypic features of *Lactococcus garvieae* and *Streptococcus iniae* isolated from different farmed rainbow trout in Iran. Results in parentheses show the published data (Austin & Austin, 1999; Savvidis et al., 2007). V = variable results; S = Sensitive; R = Resistance;? = Not defined.

Figure 1. Geographical distribution of *L. garvieae* and *S. iniae* in Iran. ● = Location of lactococcosis affected trout farms in Iran (Regions of Tehran, Mazandaran, Markazi, Kermanshah, Lorestan, Fars, Charmohal-va-Bakhteyari provinces).

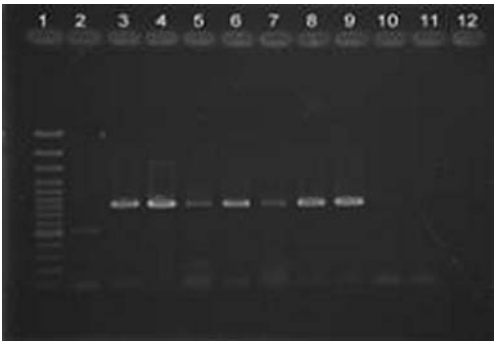

Figure 2. PCR analysis of *L. garvieae* isolated from diseased rainbow trout in Iran. Agarose gel electrophoretic analysis of PCR products of 853 bp were amplified. Lane 1 = Length marker, Lane 2 = *S. iniae* (FD strain, Iran strain) (Soltani et al., 2005), Lane 3 = *L. garvieae* (TKS KG+ Japanese strain), Lanes 4-9 = test samples of bacterial isolates from different parts of the country, Lanes 10 and 12 = Negative control (no DNA).

water sources are sometimes contaminated with warm-blooded animal sewages. This is particularly true in cases of these diseased trout farms because the upper side of these water sources is tourist areas especially during the spring and summer seasons, the time during which the highest fish mortality occurs. Therefore, it is anticipated that both *L. garvieae* and *S. iniae* have a wide host range probably including humans. Both species of these bacteria have been so far recovered from *Macrobrachium rosenbergii*, cows, buffalos and

humans (Texeira et al., 1996; Soltani et al., 2005).

Also *L. garvieae* has been isolated from poultry meat, porcine blood from industrial abattoirs and from cat and dog tonsils (Pot et al., 1996; Barakat et al., 2000; Vendrell et al., 2006). In addition the entry of new lots of fish into the fish farm is the most frequent method of introduction of *L. garvieae*. Asymptomatic carriers are the main source of infection because they carry the bacterium in their intestinal microbiota and can eliminate the bacterium in feces infecting the rest of the healthy fish in the ponds. Also, the recovered fish from the disease continue disseminating the agent for a certain period (Vendrell et al., 2006).

In a previous study by Soltani et al. (2005) *S. iniae* was reported in some trout farms in south west of Iran. In this study we also isolated not only *S. iniae* from some affected trout farms but also *L. garvieae* was frequently isolated from diseased fish. However, the rate of outbreaks by *L. garvieae* was higher in some parts of country, i.e. North East and North West (Figure 1). Because of wide distribution of rainbow trout farming in Iran and frequent transportation of larvae and fingerlings for long distances between different states, more studies are required to epizootiologically show the geographical distribution of streptococcosis and lactococcosis inside the country. This is very important because the trout production in Iran is now nearly 50000 tones and it is expected to increase to 100000 tones by year 2012.

The initial evaluation of virulence level of some isolated strains of *L. garvieae* is varied with moderate to high virulent strains and impact of poor water quality has been recognized as one of the most predisposing factors for outbreaks of Lactococcosis/ streptococcosis in Iranian farmed rainbow trout.

Our estimation of losses due to the disease outbreak in an integrated trout farm located in the south west, Charmohal-va-Bakhteyari province of Iran with a production of 2500 tones resulted in a loss of about one million US dollar during only a period of three months, during the summer of 2006. Therefore, lactococcosis/streptococcosis is becoming a serious obstacle for sustainable developing of trout industry in Iran.

Currently, the affected trout farms are using mainly erythromycin, oxytetracycline and enrofloxacin. However, the use of chemotherapeutic treatments is dramatically becoming insufficient because of increasing bacterial resistance. Also, the occurrence of granulation tissue reactions in different vital organs of brain, kidney, liver, spleen and eye of affected fish makes treatment of diseased fish unsuccessful (Kusuda & Salati, 1999; Soltani et al., 2005). Therefore, the application of strict prophylactic criteria including avoidance of importing of infected fish, frequent collection and destruction of moribund fish, regular and appropriate disinfection of equipment, improvement of health management measures and immunization of healthy fish are highly recommended in Iran trout farming industry. The vaccination of fish in some trout farms by using commercial vaccines of Schering Plough (France) has resulted in various protections probably because of heterogeneity

of involved strains in Iran region as well as poor health management after vaccination. However, use of recently produced local vaccine by University of Tehran and Jehad-Daneshgahi (Aqua Sol-1 No 008930/ 34421) resulted in a satisfactory protection.

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