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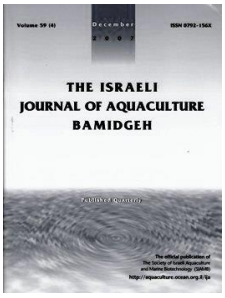

A comparative study of detection methods for *Lactococcus garvieae* in experimentally infected rainbow trout (*Oncorhynchus mykiss,* W.)

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Key words: rainbow trout, experimental infection, *Lactococcus garvieae*, histopathology, slide agglutination, Indirect Fluorescent Antibody Technique, Enzyme Linked Immunosorbent Assay

Abstract

This study was carried out to detect *Lactococcus garvieae* from experimentally infected rainbow trout (*Oncorhynchus mykiss***,** W.) using bacteriological, serological, and histopathological techniques. Rainbow trout were experimentally infected with two *Lactococcus garvieae* strains (L_1 and L_2) by intraperitonal injection. These strains caused bilateral exophthalmia with periocular hemorrhage, corneal opacification, darkening of the skin, strong internal congestion in the visceral organs, and enlargement of the spleen in the infected rainbow trout. The two *L. garvieae* strains were re-isolated from experimentally infected fish groups and identified with standard biochemical methods and API 20 STREP system. The presence of *L. garvieae* in the tissue of experimentally infected fish was confirmed by Indirect Fluorescent Antibody Technique (IFAT). The humoral antibody response of the infected fish was confirmed by slide agglutination, IFAT, and Enzyme Linked Immunosorbent Assay (ELISA). Histopathologically, liquefactive necrosis in the liver, spleen, and kidney, epithelial cells hyperplasia, hemorrhages and telangiectasia of the gill filaments, and sub-retinal hemorrhages in the eyes were observed.

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Introduction

Lactococcus garvieae has been reported to be a major pathogen of farmed fish causing significant economic losses in the aquaculture industry. The first outbreak of lactococcosis by *L. garvieae* in farmed rainbow trout occured in Spain (Palacios et al., 1993). After 1988, it was reported in farmed rainbow trout in Italy (Ghittino and Prearo, 1992), Australia and South Africa (Carson et al., 1993), England (Bark & Mcgregor, 2001) and Portugal (Pereira et al., 2004). This agent has been isolated and identified in marine fish such as grey mullet, *Mugil cephalus* (Chen et al., 2002), and flounder, *Paralichthys olivaceous* (Baeck et al., 2006). *L. garvieae* has also been identified in giant freshwater prawn, *Macrobrachium rosenbergii,* in Taiwan (Chen et al., 2001). In Turkey, *L. garvieae* was isolated and identified in diseased farmed rainbow trout from different regions of Turkey, respectively from the Aegean (Diler et al., 2002), Central Anatolia (Kav & Erganis, 2007), Mediterranean (Özer et al., 2008; Tanrıkul & Gultepe, 2010), Black Sea (Türe & Savaş, 2010) and Marmara regions (Timur et al., 2011).

Lactococcosis has been defined as a hyperacute and hemorrhagic septicemia (Bercovier et al., 1997). Typical macroscopic and clinical findings in infected rainbow trout are anorexia, lethargy, loss of orientation, exophthalmia, melanosis, hemorrhages in the ocular zone, perianal area, fin and anal prolapsus (Eldar & Ghittino, 1999).

This study describes the successful diagnosis of *L. garvieae* strains in experimentally infected fish by standard biochemical methods and the API 20 STREP system, serological methods, slide agglutination IFAT and ELISA for infected fish antisera, and IFAT for infected fish tissue. Gram staining technique of the histological section of the intestines was also applied.

Materials and Methods

Fish, Experimental İnfection, and Sampling. In the experiment which lasted 35 days, seventy (70) healthy rainbow trout (65g) were divided into three groups. A control group (20 fish) and two experimental groups (25 fish in each) were stocked into fiberglass tanks containing 1 m^3 of fresh water. Two different *L. garvieae* strains were used in the experimental infection. The first strain (L_1) was obtained from diseased farmed rainbow trout in Turkey. The second strain, ATCC-43921, (L_2) was used as a reference. Experimental infection was carried out by intraperitoneal (IP) injection of 0.1 ml of bacterial culture (10^7cells/ml) per fish in both experimental groups. The fish in the control were injected with 0.1 ml PBS. Water temperature was maintained at 17±1º C. Dissolved oxygen and pH in the water in all tanks was uniform throughout the experiment. Bacteriological, histopathological, and serological tests were carried out on moribund fish which were sampled at five day intervals during the experimental period.

Blood samples were collected from the caudal artery of moribund fish as well as from those in the control group. The antisera were prepared as described by Barnes et al. (2002) from blood samples and stored at -20° C. Tissue samples for IFAT were taken aseptically from kidney, spleen, liver, and eyes of the control and moribund fish and stored at -20° C.

Bacteriological samples from kidney, spleen and liver were streaked onto Tyriptic Soy Agar (TSA) (Merk, Darmstadt, Germany) and Brain Heart Infusion Agar (BHIA) (Merk, Darmstadt, Germany), and plates were incubated at 22° C for 48-72h. The morphological and physiological characteristics of a representative number of bacterial colonies were determined (Austin & Austin, 2012) together with their biochemical characteristics using API STREP, and identified following Bergey's Manual (Holt et al., 1994).

Samples of tissue from gut, kidney, liver, spleen, heart, gills, and eyes were fixed in 10% buffered formalin for 24h, dehydrated and embedded in paraffin. Histological sections (4µm) were stained with hemotoxylin and eosin (H&E), and Gram stained, and examined by light microscopy (Bullock, 1978).

Immunization of rabbits. Two rabbits were immunized intravenously, the first with 10^9 cells/ml of the formalin killed *L. garvieae* strains, L_1 and L_2, (Eyngor et al., 2004), and the second with polyclonal rabbit antisera obtained according to Sorensen and Larsen (1986). Inactivated bacterial cultures were prepared as described by Eyngor et al. (2004). The rabbit antisera were stored at -20° C. This protocol was approved by the Ethics Committee for Animal Experiments of the University of Istanbul.

Slide Agglutination Test. The slide agglutination test was performed using small amounts of bacterial colonies with several drops of rabbit anti-*L. garvieae* serum diluted in Phosphate Buffered Saline (PBS) and serum obtained from the infected fish groups. A control test was performed using PBS, with non-immunized rabbits and control fish sera (Toranzo et al., 1987).

IFAT for Infected Fish Tissue. 10 mm circles were smeared with sterile swabs from all the tissues on pre-cleaned slides. The smears were fixed with 100% methanol for 5 min. The fixed smears were treated with 1:200 dilution of rabbit antisera against *L. garvieae* for 30 min at 37° C, washed 3 times with PBS, incubated with 10 µl 1:100 of dilution of fluorescein isothiocyanate (FITC), labeled with goat anti-rabbit IgG (Millipore, LV1395035) for 30 min at 37° C, stained with Evans blue (0.1 %) for 30 min at 37° C and observed under the fluorescent microscope (Kang et al., 2004; Whitman, 2004; Klesius et al., 2006). A control test was performed with tissues from the control group.

IFAT for Infected Fish Antisera. L. garvieae antigens were prepared according to Barnes et al. (2002) and used as the test antigen for IFAT. Drops of antigen (10^9 cell/ml) were placed on a slide, fixed with 100% acetone for 10 minutes, incubated with undiluted antisera taken from moribund fish, and later incubated with 1:4000 dilution of rabbit anti-salmonid Ig (RayBiotech, DS-PB-02926) for 30 min at 37° C. Slides were washed 3 times with PBS. The slides were then treated with 1:100 dilution of FITC labeled with goat anti-rabbit IgG for 30 min at 37° C, washed 3 times with PBS, and stained with Evans blue (0.1 %) 30 min at 37° C. 100 µl of 25% glycerol solution was added and observed under the fluorescent microscope (Lewis and Savage, 1972; Anderson, 1990; Austin and Austin, 2012). A control test was performed with the control fish sera.

Enzyme Linked Immunosorbent Assay. ELISA plates with 96 wells were coated overnight at 4ºC with 100 µl of formalin-free *L. garviaeae* suspension (diluted in carbonate/bicarbonate buffer pH 9.6 (Sigma, 019K8207) at OD_{630}:0.6), washed 3 times with PBST (PBS containing 0.05 % Tween 20) and blocked with blocking solution (PBS containing 3% Bovine Serum Albumin) at 25ºC for 1h. The plates were washed and serum samples in blocking buffer, obtained from moribund fish, were added at different dilutions: 1:10, 1:20, 1:50, and 1:100 for determining the specific antibody response to *L. garvieae*. After incubation, plates were washed as described above, polyclonal rabbit anti-salmon Ig was added, incubated at 25º C for 1h, and washed three times with PBST. 100 µl peroxidase conjugated goat anti-rabbit IgG (Invitrogen) was added to each well and plates were incubated for 1 h at 25 C. Unbound conjugated goat anti-rabbit IgG was washed off and 50 µl substrate (0.04 % o-phenylenediamine dihydrochloride and 0.04 % H_2O_2 of sodium phosphate-citrate buffer (Sigma, 019K8206) was added to each well. The enzymatic reaction was stopped by adding 50 µl 3 N H_2SO_4 to each well. The optical density was measured at 450 nm.

Results

Experimental and Clinical Findings. First clinical signs such as loss of orientation and exophthalmia were observed 2 days after an IP injection of L_1 and L_2. In addition, first fish mortalities were observed in both experimental fish groups 5 days after injection of *L. garvieae* strains. The comparison of cumulative mortality of rainbow trout challenged with *L. garvieae* strains (L_1 and L_2) is given in fig. 1. Mortality rate from L_1 and L_2 strains reached 96% and 88% respectively in 35 days.

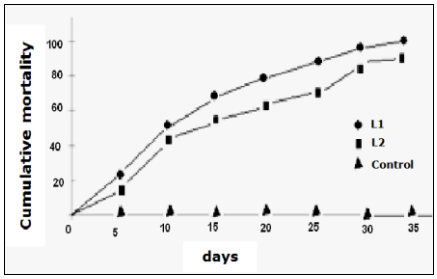
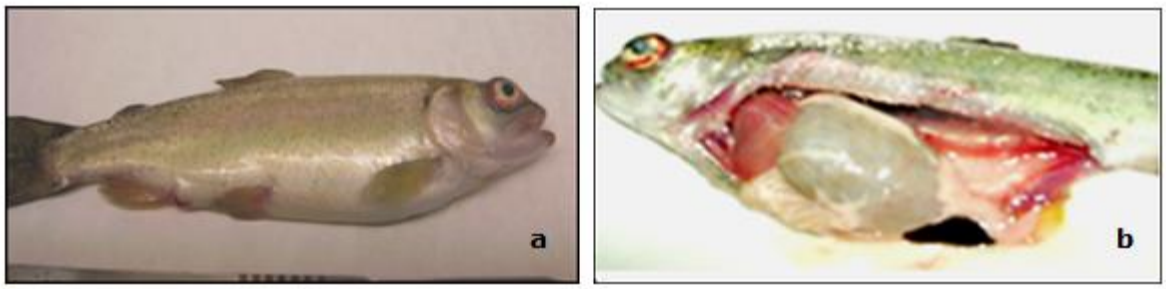


Fig. 1. Comparison of cumulative mortality of rainbow trout challenged with L. garvieae strains (L_1 and L_2)

Experimentally infected fish showed externally bilateral exophthalmia with periocular hemorrhage (Fig. 2a) and corneal opacification, darkening of the skin, hemorrhages at

the base of fins, perianal area, and enlarged abdomen. Internally they had severe congestion in the liver, intestine, and swim bladder, yellow gelatinous fluid in the intestine, and enlargement of the spleen (Fig. 2b) in both experimentally infected fish groups. No clinical signs were observed in the control group.

Fig. 2a. Periocular hemorrhages, hemorrhages at the base of fins, perianal area and enlarged abdomen (10 days after injection); 2b. Congestion in the liver, intestine and swim bladder, yellow gelatinous fluid in the intestine, enlargement of the spleen (15 days after injection)



Bacteriological findings. Mortality was considered to be a result of the inoculated *Lactococcus* strains if the bacterium was recovered in pure culture from internal organs such as kidney, spleen, and liver. After incubation of kidney, spleen, and liver swabs, white colonies of *L. garvieae* were observed. The bacteria were non-motile, capsulated, Gram positive cocci, oxidase and catalase negative, alfa hemolitic on blood agar. According to biochemical test results (Table 1) and API 20 STREP, re-isolates were identified as *Lactococcus garvieae*. No other pathogenic bacteria were isolated from either infected fish groups.

Table 1. Biochemical test results of re-isolates (L_1 and L_2) from experimentally infected rainbow trout

Characteristics		*L1*	*L2*
Gram staining		+	+
Indian ink capsule stanning		+	+
Catalase		-	-
Oxidase		-	-
Motility		-	-
O/F		F	F
5% Blood Agar		α	A
VP		+	+
Indole		-	-
Arginine		+	+
Ornithine		-	-
Lysine		-	-
MR		+	+
β-galactosidase		-	-
	D-glucose	+	+
	L-arabinose	-	-
	D-xylose	-	-
	D-mannose	+	+
	D-cellobiose	+	+
	D- galactose	-	-
	Gelatin	-	-
	Starch	-	-
	Aesculin	+	+
Utilization of citrate		+	+
Urease		-	-
	20 °C	+	+
	37 °C	+	+
	45 °C	+	+
	1.5% NaCl	+	+

Characteristics		
Gram staining	+	+
Indian ink capsule stanning	+	+
Catalase	-	-
Oxidase	-	-
Motility	-	-
O/F	F	F
5% Blood Agar	α	α

VP	+	+
Indole	-	-
Arginine	+	+
Ornithine	-	-
Lysine	-	-
MR	+	+
β-galactosidase	-	-
D-glucose	+	+
L-arabinose	-	-
D-xylose	-	-
D-mannose	+	+
D-cellobiose	+	+
D- galactose	-	-
Gelatin	-	-
Starch	-	-
Aesculin	+	+
Utilization of citrate	+	+
Urease	-	-
20 °C	+	+
37 °C	+	+
45 °C	+	+
1.5% NaCl	+	+
6.5% NaCl	+	+

+: positive -: negative F: fermentative α: alfa hemolitic

Histopathological findings. Histopathologically, multifocal liquefactive necrosis in the spleen (Fig. 3a) and kidney were observed. Tubular epithelium degeneration and periglomerular oedema was present in the kidney (Fig. 3b). Degeneration of hepatocytes or multifocal liquefactive necrosis and oedama in the liver (Fig. 3c), hemorrhages, talengectiasis and hyperplasia in the gill epithelium (Fig. 3d), sub-retinal hemorrhage in the eyes (Fig. 3e) were also observed. In addition to these findings Gram positive bacteria were observed in the lamina propria of intestine (Fig. 3f).

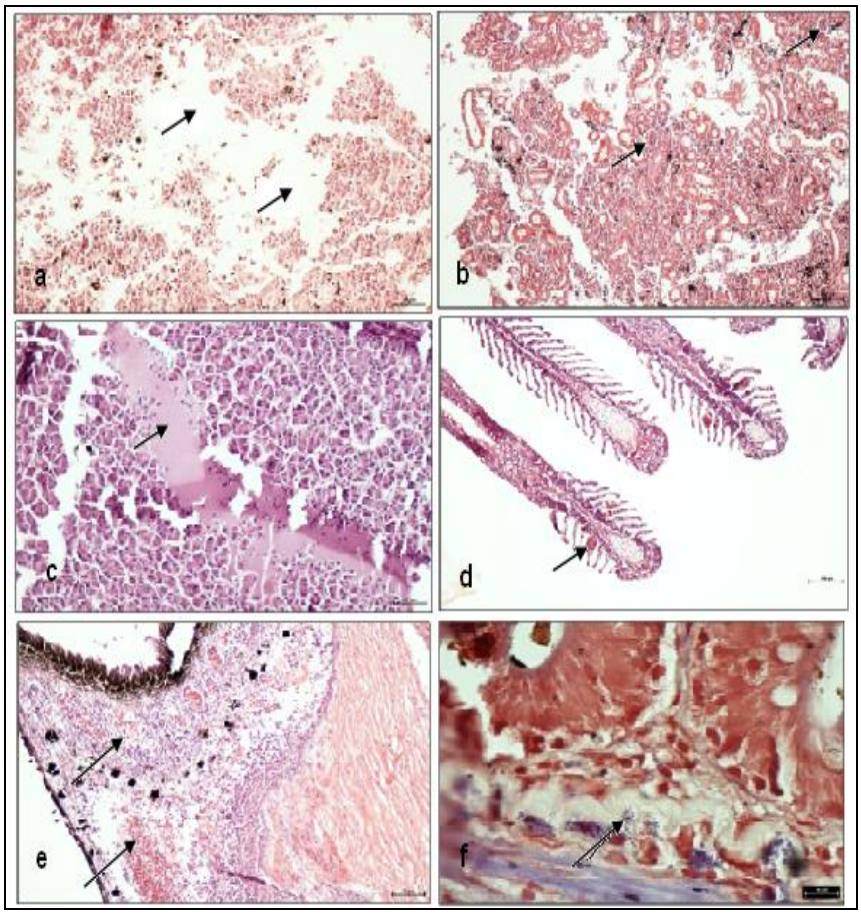


Fig. 3a. Multifocal liquefactive necrosis (arrow) in the spleen (10 days after injection);
3b. Multifocal liquefactive necrosis, periglomerular oedema (arrow), degeneration of the tubular epithelium in the kidney (15 days after injection);
3c. Liquefactive necrosis (arrow) and oedema in the liver (15 days after injection);
3d. Multifocal hemorrhage, talengectiasis (arrow) and hyperplasia of the gill epithelium (15 days after injection)
3e. Sub-retinal hemorrhage in the eye (arrow) H&E (15 days after injection);
3f. Ovoid shaped Gram positive bacteria in the intestinal lamina propria (arrow), Tissue Gram stained (10 days after injection)

Serological findings. The slide agglutination test provided a positive reaction against fish antisera from both infected fish groups but strong agglutination was observed in the blood serum 30 days after injection. Settling patterns were examined and scored (Table 2).

Table 2. Slide agglutination test result

After injection	*Agglutination*
5 days later	-
10 days later	-
15 days later	-
20 days later	+
25 days later	++
30 days later	+++
35 days later	+++

(++++ complete agglutination; +++ strong agglutination; ++ moderate agglutination; + slight agglutination; - no agglutination)

IFAT detected and identified L_1 and L_2 in the eye, spleen, kidney, and liver samples obtained from moribund fish between 5 and 35 days post-challenge. The lactococcosis organism showed bright fluorescence with IFAT. While bacteria with green fluorescence were most abundant in the eyes and spleen tissue, these were found in low numbers in the kidney and liver (Fig 4a). IFAT also showed the presence of specific antibody in the blood serum; antibody and antigen complex showing green fluorescence were observed in moribund fish 30 days after injection (Fig 4b). IFAT correctly determined L_1 and L_2 strains from both serum and tissue.

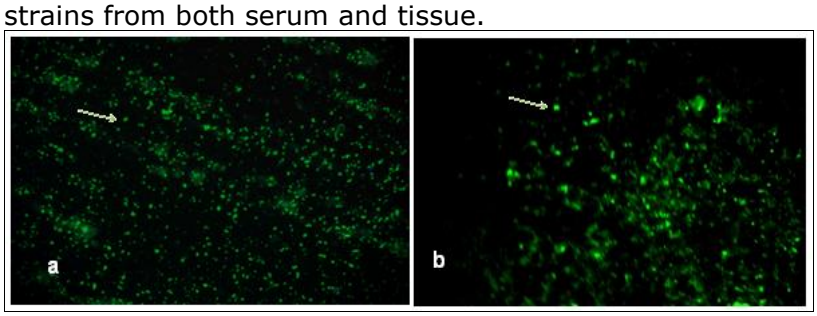


Fig. 4a. Presence of coccus bacteria with green fluorescence (arrow) under the fluorescent microscope (eye tissue) x200; 4b. Antibody & antigen complex fluoresced brilliantly (arrow) in the serum x400

The specific antibody response to L. garvieae strains was measured using ELISA. This showed significant serum antibody response. The antibody response of both experimentally infected fish groups was significantly different from the non-infected rainbow trout. The presence of specific antibodies against L_1 and L_2 strains in the serum were clearly seen 30 days following experimental infection by ELISA.

Discussion

Since the first discovery of *Lactococcus garvieae* in rainbow trout in 1988 in Spain, the presence of *L. garvieae* in fish and fresh water prawn has become increasingly apparent. The incidence of mortality due to lactococcosis has increased dramatically (Ghittino and Prearo, 1992; Bark and Mcgregor, 2001; Diler et al., 2002; Pereira et al., 2004; Kav and Erganis, 2007; Timur et al., 2011). The losses incurred can exceed 50-80% of total rainbow trout production (Ghittino and Prearo, 1992).

Experimental infection induced by intraperitonal injection of *L. garvieae* in yellowtail (*Seriola quinqueradiata*) resulted in disease symptoms appearing 2-3 days after injection (Itami et al. 1996). In grey mullet infected by intramuscular injection disease symptoms appeared after 2 days resulting in 100% mortality (Chen et al., 2002). In other studies on rainbow trout, first signs of disease as well as mortality were observed 2-3 days after injection (Muzquiz et al., 1999; Avci et al., 2013). In our study first clinical signs were observed 2 days after IP injection in rainbow trout but mortalities began 5 days after injection in the both experimental fish groups. Farmed rainbow trout naturally infected with *L. garvieae* had a cumulative mortality of approximately 80% (Diler et al. 2002). In our study mortality rate was 96% and 84% in L_1 and L_2 strains respectively. This result showed that these bacteria require a short incubation period in fish tissue with a sudden onset induced hyperacute systemic disease. In our study, fish

infected with *L. garvieae* demonstrated clinical signs and gross pathology similar to those described by Eldar and Ghittino (1999) and Muzquiz et al., (1999).

Histopathologically, multifocal liquefactive necrosis and hemorrhages in the kidney, spleen and liver, periglomerular oedema, and interrenal hemopoietic tissue in the spleen, sloughing of the necrotic mucosa epithelium to the intestinal lumen, and the presence of ovoid shaped Gram positive bacteria in the intestinal lamina propria were observed in experimentally infected fish as well as in naturally infected rainbow trout (Timur et al., 2011). Although, in another experimental study, oedema and swelling were seen in the secondary lamellar epithelium in the gills (Avci et al. 2013), in our study multifocal hemorrhages, talengectiasis, and hyperplasia of the gill epithelium were observed in the gills. Focal or multifocal coagulation necrosis and oedema have been found in the spleen (Avci et al. 2013), but in our study only multifocal liquefactive necrosis and hemorrhages were observed in the spleen. Degeneration of the tubular epithelium, with hyalin droplets were not observed in the kidneys (Avci et al. 2013) but we observed degeneration of tubular epithelium without hyalin droplets as well as multifocal liquefactive necrosis in the kidney tissue. Pycnotic nuclei and disrupted cytoplasm were found along with the degeneration of hepatocytes (Avci et al. 2013). In contrast to these findings, we observed multifocal liquefactive necrotic areas and hemorrhages in the liver. In addition to these histopathological findings acute meningitis was found in the experimentally infected rainbow trout (Eldar and Ghittino 1999) as well as multifocal petechial hemorrhages in the viceral organs (Altun et al. 2007). The presence of ovoid shaped Gram positive bacteria in the eye tissues was also found (Avcı et al. 2010; Timur et al. 2011).

Antibodies, both monoclonal and polyclonal have been widely used for diagnosis of fish diseases. Several antibody-based methods have been used including slide agglutination, direct and indirect fluorescent antibody tests, and ELISA for identification of fish pathogens (Bakopoulos et al., 1997; Ooyama et al., 2002; Kang et al.,2004; Klesius et al., 2006).

The slide agglutination test provided rapid detection of *L. garvieae* strains (L_1 and L_2) that were divided into KG (-) and KG (+) phenotype cells with anti-KG (+) sera (Yoshida et al., 1997; Ooyama et al., 2002). According to the agglutination test described by Yoshida et al. (1997) and Ooyama et al. (2002), L_1 and L_2 strains found were KG (+) phenotype cells because L_1 and L_2 strains agglutinated with anti-KG (+) sera. This morphological structure was confirmed with the Indian ink capsule staining method.

In this study, green fluorescent colored, ovoid shaped bacteria were observed in different tissues of both groups of experimentally infected rainbow trout by using IFAT (Kang et al. 2004). Fluorescent labeling of *L. garvieae* antigen were observed in abundance in the kidney, spleen, and heart tissue of infected fish using immunohistochemical staining methods (immunofluorescence) (Avci et al. 2013). However, using IFAT we observed many ovoid shaped pathogens in the eye and spleen tissues, but found fewer in the kidney and liver tissues. This result supported the previous reports about the location of this pathogen in fish tissues (Carson et al., 1993; Savvidis et al., 2007).

Green fluorescent antigen-antibody complex was used to determine different fish pathogens such as *Aeromonas liquetaciens* (Lewis and Savage, 1972), *Flexibacter psychrophilus* (Lorenzen and Karas, 1992), and *Renibacterium salmoninarum* (Anderson, 1990) by IFAT. However there is no information regarding the determination of *L. garvieae* from infected fish sera with IFAT. In this study, green fluorescent colored antigen-antibody complex was first successfully determined by infected fish sera obtained from experimentally infected fish groups using IFAT.

ELISA has been used in several studies of the humoral antibody response in fish. It is a sensitive method which permits rapid screening of a large number of serum samples for Gram negative bacteria (Austin et al., 1986; Cossarini-Dunier, 1985; Bakopoulos et al., 1997). Nowadays ELISA is used to determine antibody production immunized by Gram positive bacteria such as *Streptococcus iniae* (Shelby et al., 2002)

and *S. agalactiae* (Pasnik et al., 2006). No information is available describing an immunoassay for antibody response against *L. garvieae* in rainbow trout. In this study, the presence of specific antibodies against L_1 and L_2 strains in the infected fish serum were clearly seen 30 days following IP infection by ELISA.

The assay described in this study was developed with the aim of providing a detection method for *L. garvieae* that would be more rapid and sensitive. Bacteriological and serological findings were confirmed using a histopathological technique. All serological techniques have a shorter analysis time, use much less serum and non-lethal methods to detect antibodies to *L. garvieae* strains. Development and use of this assay will expedite the detection of antibodies to *L. garvieae,* which will improve the monitoring of vaccine responses and/or natural exposure to *L. garvieae*. This could possibly be done in the early stages of *L. garvieae* epizootics in order to take preventive measures and protect the overall population.

Acknowledgements

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References

Altun, S., Adiloğlu, A.K., Kubilay, A., Diler, Ö., Demirbaş, N. and R. Sütçü 2007. Immunogenic and antigenic profiles of nine *Lactococcus garvieae* strains from different rainbow trout farms, *Isr. J. Aquacult.- Bamidgeh,* 59(2): 111-116.

Anderson, D.P. 1990. Fluorescent Antibody Test, in *Fish Immunology Technical Communications-1 (FITC).* Eds. J.S. Stolen, T.C. Fletcher, D.P. Anderson, B.S. Roberson and W.B. Van Muiswinkel, pp.1-8. SOS Pub, USA.

Austin, B., Bishop, I., Gray, C., Watt, B. and J. Dawes 1986. Monoclonal antibody-based enzyme linked immunosorbent assays for the rapid diagnosis of clinical cases of enteric redmouth and furunculosis in fish farms. *J. Fish Dis.,* 9: 469-474.

Austin, B. and D. Austin, 2012. *Bacterial Fish Pathogens Disease of Farmed And Wild Fish*, Fifth Ed., pp. 38-42. Springer Dordrecht Berlin Heidelberg New York.

Avcı, H., Aydoğan, A., Tanrıkul, T.T. and S.S. Birincioğlu 2010. Pathological and microbiological investigations in rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) naturally infected with *Lactococcus garvieae. Kafkas Universitesi Veteriner Fakültesi Dergisi,* 16: 313-318.

Avci, H., Birincioglu, S. S., Tanrikul, T. T., Epikmen, E. T., Metin, N. and M. L. Avsever 2013. Experimental *Lactococcus garvieae* infection in rainbow trout, *Oncorhynchus mykiss*, Walbaum 1792: a comparative histopathological and immunohistochemical study. *J. Fish Dis.*, doi:10.1111/jfd.12132 (in press)

Baeck, G.W., Kim, J.H., Gomez, D.K. and S.C. Park, 2006. Isolation and characteization of *Streptococcus* sp. from diseased flounder (*Paralichthys olivaceus*) in Jeju Island. *J. Vet. Sci.,* 7: 53-58.

Bakopoulos, V., Volpatti, D., Papapanagiotou, E., Richard, R., Galleotti, M. and A. Adams 1997. Development of an ELISA to detect *Pasturella piscicida* in culture and in fish tissue. *Aquaculture,* 156:359-366.

Bark, S. and D. Mcgregor, 2001. The first occurrence of lactococcosis in farmed trout in England. *Trout News,* 31:9-11.

Barnes, A., Guyat, C., Hansen, B., Horne, M. and A. Ellis, 2002. Antibody increases phagocytosis and killing of *Lactococcus garvieae* by rainbow trout (*Oncorhynchus mykiss)* macrophages. *Fish Shellfish Immun.,* 12:181-186.

Bullock, A.M. 1978. *Laboratory Methods in Fish Pathology* (RJ Roberts Ed.) pp. 235-267. Bailliere Tindall, London.

Carson, J., Gudkovs, N. and B. Austin, 1993. Characteristics of an *Enterococcus-like* bacterium from Australia and South Africa, pathogenic for rainbow trout, *Oncorhynehus mykiss* (Walbaum). *J. Fish Dis.,* 16:381-388.

Chen, S.C., Liaw, L.L., Su, H.Y., Ko, S.C., Wu, C.Y., Chaung, H.C., Tsai, Y.H., Yang, K.L., Chen, Y.C., Chen, T.H., Lin, G.R., Cheng, S.Y., Lin, Y.D., Lee, J.L., Lai, C.C., Weng, Y.J. and S.Y. Chu, 2002. *Lactococcus garvieae*, a cause of disease in grey mullet, *Mugil cephalus* L., in Taiwan. *J. Fish Dis.,* 25:727-732.

Chen, S.C., Lin, Y.D., Liaw, L.L. and P.C. Wang 2001. *Lactococcus garvieae* infection in the giant freshwater prawn *Macrobranchium rosenbergii* confirmed by polymerase chain reaction and 16S rDNA sequencing. *Diseases of Aquatic Organisms,* 45: 45-52.

Cossarini-Dunier, M. 1985. Indirect enzyme-linked immunosorbenl assay (ELISA) to titrate rainbow trout serum antibodies against two pathogen: *Yersinia ruckeri* and Egtved virüs. *Aquaculture,* 49:197-208.

Diler, Ö., Altun, S., Adiloğlu, A., Kubilay, A. and B. Işıklı, 2002. First Occurance of Streptococcosis affecting farmed rainbow trout in Turkey. *Bulletin of the European Association of Fish Pathologists,* 22:21-26.

Eldar, A. and C. Ghittino, 1999. *Lactococcus garvieae and Streptococcus iniae* infection in rainbow trout similar; but different disease. *Dis. Aquat. Organ.,* 36:227-231.

Eyngor, M., Zlotkin, A., Ghittino, C., Prearo, M., Douet, D.G., Chilmonczyk, S. and A. Eldar, 2004. Clonality and diversity of the fish pathogen *Lactococcus garvieae* in Mediterranean countries. *Appl. Environ. Microb.,* 70:5132-5137.

Ghittino, C. and M. Prearo, 1992. Report of Streptococcosis in rainbow trout (*Oncorhynchus mykiss*) in Italy. *Bollettıno Della Societa Paleontologica Italiana,* 8: 4-11.

Holt, J., Krieg, N.R., Sneath, P.H., Staley, J.I. and S.T. Williams 1994. *Bergey's Manual of Determinative Bacteriology.* pp:787. Ninth Edition. Williams&Wilkins.

Itami, T., Kondo, M., Uozo, M., Suganuma, A., Abe, T., Nakagawa, A., Suzuki, N. and Y. Takahashi, 1996. Enhancement of resistance against *Enterococcus seriolicida* infection in yellowtail, *Seriola quinqueradiata*, by oral administration of peptidoglycan derived from *Bifidobacterium thermophilum. J. Fish Dis.,* 19:185-187.

Kang, S., Shin, G., Shin, Y., Kim, Y., Yang, H., Lee, E., Huh, N., Ju, O. and T. Jung, 2004. Experimantal evaluation of pathogenicity of *Lactococcus garvieae* in black rockfish (S*ebastes schlegeli*). *J. Vet. Sci.,* 5: 387-390.

Kav, K. and O. Erganis, 2007. Konya Bölgesinde bulunan (*Oncorhynchus mykiss*) çiftliklerinden *Lactococcus garvieae* izolasyonu identifikasyonu ve fenotipik özelliklerinin belirlenmesi. *Veteriner Bilimleri Dergisi,* 23: 7-17.

Klesius, P., Evans, J., Shoemaker, C., Yeh, H., Goodwin, A.E., Adams, A. and K. Thompson, 2006. Detection and İdentification of *Streptococcus iniae* using a monoclonal antibody based indirect fluorescent antibody technique. *Aquaculture,* 258:180-186.

Lewis, D.L. and N.L. Savage, 1972. Detection antibodies to *Aeromonas liquefaciens* in fish by an Indirect Fluorescent Antibody Technique. *J. Fısh. Res. Board of Canada,* 29: 211-212.

Lorenzen, E. and N. Karas, 1992. Detection of *Flexibacter psychrophilus* by immunofluorescens in fish suffering from fry mortalite syndrome: a rapid diagnostic method. *Dis. Aquat. Organ.,* 13: 231-234.

Muzquiz, J.L., Royo, F.M., Ortego, C., Blas, I., Ruiz, I. and J.L. Alonso, 1999. Pathogenicity of *Streptococcosıs in* Rainbow trout *(Oncorhynchus mykıss):* Dependence of age and diseased fish. *Bull. Eur. Assoc.f Fish Pathol.,* 19: 114-119.

Ooyama, T., Hirokawa, Y., Minami, T., Yasuda, H., Nakai, T., Endo, M., Ruangpan, L. and T. Yoshida, 2002. Cell-surface properties of *Lactococcus garvieae* strains and their immunogenicity in the yellowtail *Seriola quinqueradiata*. *Dis. Aquat. Organ.,* 51: 169-177.

Özer, S., Bulduklu, S. and E. Dönmez, 2008. Streptococcocis occurence at rainbow trout (*Oncorhynchus mykiss*, Walbaum) cultivated in province Mersin-Turkey. *J. Fish. Sci.,* 2: 272-283.

Palacios, M.A., Zamora, M.J., Vasquez, J., Zamora, E. and A. Duran, 1993. Streptococcosis in rainbow trout (*Oncorhynchus mykiss*) in Spain. *Bollettıno Della Societa Paleontologica Italiana,* 13: 11-16.

Pasnik, D.J., Evans, J.J. and P.H. Klesius, 2006. Passive İmmunization of Nile Tilapia (*Oreochromis niloticus)* provides significant protection aganist *Streptococcus agalactiae*. *Fish Shellfish Immun.,* 21: 365-371.

Pereira, F., Ravelo, C., Toranzo, A.E. and J.L. Romalde, 2004. *Lactococcus garvieae*, an emerging pathogen for the Portuguese trout culture. *Bull. Eur. Assoc. Fish Pathol.,* 24: 274-279.

Savvidis, G.K., Anatoliotis, C., Kanaki, Z. and G. Vafeas, 2007. Epizootic outbreak of Lactococcosis disease in rainbow trout culture in Greece. *Bull. Eur. Assoc. Fish Pathol.,* 27: 223-228.

Shelby, R.A., Klesius, P.H., Shoemaker, C.A. and J.J. Evans, 2002. Passive immunization of tilapia, *Oreochromis niloticus* (L.), with anti-*Streptococcus iniae* whole sera. *J. Fish Dis.,* 25: 1-6.

Soltani, M., Nikbakht, G., Mousavi, H.A.E. and N. Ahmadzadeh, 2008. Epizootic outbreaks of lactoccocosis caused by *Lactococcus garvieae* in rainbow trout in Iran. *Bull. Eur. Assoc. Fish Pathol.,* 28: 207-212.

Sorensen, U.B.S. and J.L. Larsen, 1986. Serotyping of *Vibrio anguillarum*. *Appl. Environ. Microb.,* 51: 593-597.

Tanrıkul, T.T. and N. Gültepe, 2011. Mix Infection in Rainbow Trout (*Oncorhynchus mykiss* Walbaum*) Lactococcus garvieae* and *Vibrio anguillarum* O1. *J. Anim. Vet. Adv.,* 10: 1019-1023.

Timur, G., Yardımcı, R.E., Ürkü, Ç. and Ö. Çanak, 2011. Marmara Bölgesi kültür alabalıklarında (*Oncorhynchus mykiss*, L.) lactococcosis' in bakteriyolojik ve histopatolojik metodlarla teşhisi, İ.Ü. *Su Ürünleri Fakültesi Dergisi,* 26:63-81.

Toranzo, A.E., Baya, A., Roberson, B., Barja, J., Grimes, D. and F. Hetrick, 1987. Specificity of slide agglutination test for detecting bacterial fish pathogen. *Aquaculture,* 61: 81-97.

Türe, M. and H. Savaş, 2010. Karadeniz Bölgesinde gökkuşağı alabalıklarında (*Oncorhynchus mykiss)* lactococcosis (*L. garvieae*). *Yunus Araştırma Bülteni,* 3: 19-20.

Whitman, K.A. 2004. *Finfish and Shellfish Bacteriology Manual Techniques and Procedures*. Blackwell Publishing Company.

Yoshida, T., Endo, M., Sakai, M. and V. Inglis, 1997. A cell capsule with posible involvement in resistance to opsonophagocitosis in *Enterococcus seriolicida* isolated from yellowtail *Seriola quinqueradiata*. *Dis. Aquat. Organ.,* 29: 233-235.