J. Vet. Sci. (2004), **5**(4), 387–390

JOURNAL OF
Veterinary
Science

Short Communication

Experimental evaluation of pathogenicity of *Lactococcus garvieae* in black rockfish (*Sebastes schlegeli*)

Sung-hyun Kang[1], Gee-wook Shin[1], Yong-seung Shin[1], Palaksha K. J.[1], Young-rim Kim[1], Hyang-hee Yang[1], Eun-young Lee[1], Eung-goo Lee[1], Nam-eung Huh[1], Oh Myung Ju[2], Tae-sung Jung[1,*]

[1]*Institute of Animal Medicine and College of Veterinary Medicine, Gyeongsang National University, Jinju 660-701, Korea*
[2]*Department of Fish Pathology, Yosu National University, Yosu 550-747, Korea*

Black rockfish (*Sebastes schlegeli*) is an important mariculture species in Korea. The production of this fish is drastically declined due to bacterial diseases, particularly streptococcosis caused by *Lactococcus garvieae*. The bacterial surface characteristics of SJ7 and TY6 were found to have capsule but not NB13 and YS18. The experiential evaluation of *L. garvieae* pathogenicity, the capsular isolates showed high cumulative mortality i.e. SJ7 (100%) and TY6 (60%) compared to non-capsular isolates. Based on this result the capsular isolates *L. garvieae* were highly suspected as the causative agent of streptococcosis in rockfish.

Key words: *Lactococcus garvieae*, Black rockfish (*Sebastes schlegeli*), mariculture

Aquaculture, especially mariculture, has become one of the major food producing sectors in Korea [1,14,16]. Among mariculture fishes, black rockfish is a very important species in the point of view of consumer's preference and as well as its compatibility with flounder in polyculture. The production of black rockfish was recorded up to 16,548 M/T, contributing about 45% of the total mariculture fish production in Korea (2002 Statistics, Ministry of Maritime Affairs & Fisheries, Korea).

Since a decade the mariculture industry in Korea is facing a serious problem of outbreak of several bacterial diseases, particularly, streptococcosis. *Lactococcus garvieae* has been reported as one of the major causative agent of streptococcosis or lactococcosis in fish [2,3]. The ubiquitous nature (sediment, water) and capability of horizontal transmission, the pathogen has become real biological threat to development of black rock fish farming industry. It is also reported to infect fishes [4,9,10,15,19,20,22], avian [5], water buffalo [21], and human [11,12,13].

L. garvieae infected fishes exhibit a variety of clinical signs, such as anorexia, exophthalmia, melanosis, conjunctivitis, erect swimming, severe internal hemorrhage and congestion of blood vessel, peritonitis, abscess of spleen and liver, meningoencephalitis, and bacterial septicemia [4,9,10,15]. The similar clinical signs were also reported from the fish farms subjected to disease outbreak due to *L. garvieae* infection. In this context, the present study was carried out to evaluate the pathogenicity of *L. garvieae* isolated from the diseased olive flounder (*Paralichthys olivaceus*) to rockfish.

The four isolates of *L. garvieae* (Table 1) were isolated from diseased yellowtail and olive flounder collected from different locations in Korea. Isolates were stored in Tryptone Soya Broth (TSB; Oxoid, England) with 0.5% yeast extract (BD, Sparks, USA) and 20% glycerol at −80°C until use. *L. garvieae* strain KG (-) 9408 was kindly provided from Dr. Ooyama, Japan and stored as the above. The pathogenicity of bacteria in combination with electronic microscopic studies revealed that the capsular envelop of *L. garvieae* KG (-) was more virulence than either micro- or non-capsular *L. garvieae* KG (+) [18]. All the Korean isolates in the present study were identified as *L. garvieae* with API® 20 Strep kit (BioMereux, France) and Polymerase Chain Reaction (PCR) with primers specific for *L. garvieae* 16S rDNA sequence [6,9,21,23]. After thawing, the bacteria was inoculated to Tryptic Soy Agar (TSA; BD) and incubated at 25°C for 24 hrs, and the bacterial colonies on TSA were

Table 1. Isolates and type strains of *L. garvieae* used in challenge experiments

Strain number	Hosts	Year of isolation	Country
SJ7	Flounder	2001	Korea
TY6	Flounder	2001	Korea
NB13	Flounder	2001	Korea
YS18	Yellowtail	2001	Korea
KG (-) 9408	Yellowtail	1998	Japan

*Corresponding author
Tel: +82-55-751-5822; Fax: +82-55-751-5803
E-mail: jungts@gsnu.ac.kr

transferred to TSB and incubated for overnight at 25°C. This broth containing *L. garvieae* was used for challenge experiment and antibody production.

Agglutination test was employed to characterize *L. garvieae* isolates and compared the existence of capsule between the isolates with the antisera raised against the *L. garvieae* strains KG (-) and KG (+). The test was performed using microplate assay developed by Ooyama *et al.* [18]. One hundred µl of bacterial broth culture (1×10^9 CFU ml^{-1}) was washed with PBS twice and distributed in 96 well plates. This plate was then incubated 50ìl of rabbit anti-KG (-) 9408 and anti-KG (+) sera (1 : 50) by two fold for 1 hr at 37°C or overnight at 4°C. The results were recorded by comparing with control, the bacteria incubated with PBS instead of antisera.

Black rockfish of average weight 20 g were purchased from a commercial fish farm located at Nam-hea County, and then transferred to fish challenge room. These fishes were divided into 5 groups 10 each and acclimatized to room condition in the tank with 10L of seawater in flow-through system for three days and fed with commercial pellet. After treating the fishes with anesthesia AQUI-S® (5% Isoeugenol, 0.3 ml/L; AQUI-S New Zealand LTD, New Zealand), each group of rockfish was injected intraperitoneally (IP) with 100ìl of four *L. garvieae* isolates (1×10^7 CFU ml^{-1}) and control group with sterile PBS. The fishes were maintained at 18°C, and monitored for clinical signs and mortality for 25 days. The moribund and dead fishes were collected and subjected to indirect fluorescent antibody test (IFAT) and PCR to identify the bacterial isolates.

The broth cultures of *L. garvieae* isolate KG (-) 9408 was centrifuged and the pellet resuspended in PBS, inactivated with formalin to a final concentration of 0.3% and stored at 4°C for 24 hr. The suspension was washed with PBS and adjusted by spectrophotometer as optical density (O.D.) 1.0 at 540 nm corresponding to 1×10^9 CFU ml^{-1}. Five month old chickens were immunized subcutaneously (S.C.) on legs with 1×10^9 CFU of the formalin killed *L. garviae.* The bacteria were mixed with Freunds complete (1st immunization) and incomplete adjuvant (2nd and 3rd immunization) and injected at 2-week-interval, and a booster dose was given after 2 weeks. After 8 weeks of immunization, chicken IgY was purified from chickens eggs using EGGstract® IgY purification kit (Promega, USA). The purified chicken IgY was stored at –20°C until use.

Moribund/dead fishes were dissected and collected either spleen or head kidney, cut with sharp scalpel, stamped the cut side on cleaned slide, and fixed it with 100% methanol for 5 min. The stamped area was marked with nail banish and incubated with 1 : 200 of dilution of chicken IgY raised against *L. garvieae* for 30 min at 37°C, washed the slide with PBS three times, incubated with 10 µl of fluorescein isothiocyanate (FITC) labelled anti-chicken IgY (Jackson ImmunoResearch Laboratories, USA) diluted (1 : 100) in humidity chamber for 30 min at 37°C. After washing the slide thoroughly with PBS, 100 µl of 25% glycerol solution was added and observed under the fluorescent microscope (Axioskop, Karl-zeiss, Germany) for bacteria.

PCR technique was also used to identify *L. garvieae* bacteria isolated from moribund/dead fishes [23]. The bacterial DNA was extracted by adding 20 µl of Gene releaser (Bioventures, USA) in accordance with manufacturer's protocol. The amplification of the extracted DNA of the isolates was carried out by *L. garvieae* 16S rDNA gene specific primers, pLG-1 (5'-CATAAC AATGAG AATCGC-3') and pLG-2 (5'-GCACC CTCGC GGGTTG-3') with programmable heating incubator (PTC-100, MJ Research Inc., USA). The amplification steps include initial denaturation at 94°C for 5 min, followed by 35 cycles of each consisting of a denaturation at 94°C for 30 sec, annealing at 55°C for 1 min, extension at 72°C for 45 sec plus final extension for 5 min at 72°C after 35 cycles. The amplified product was maintained at 4°C for 5min using kit (AccuPower® HL PCR PreMix, Bioneer, Korea) and electrophoresed in 1.2% agarose gel at 50 V till the dye reaches the end of the gel. The specific bands on mini-gel apparatus were recorded by Polaroid image capture with ECL™ mini camera (Amersham Bioscience, Sweden). DNA from KG (-) 9408 was used as positive control. *Streptococcus iniae* was used as negative control.

In the agglutination test, except one out of four bacterial isolates (TY6), all other isolates (SJ7, NB13 and YS18) were agglutinated by rabbit anti-KG (-) sera at the dilution of 1 : 100, 1 : 200, and 1 : 800, respectively. Isolates NB13 and YS18 were also agglutinated with rabbit anti-KG (+) sera at the dilution of 1 : 1,600 and 1 : 200, respectively, whereas the isolates SJ7 and TY6 could not agglutinate with rabbit anti-KG (+) sera. Agglutination test was very useful to characterize phenotype of *L. garvieae* since the phenotype of the pathogens were divided into KG (-) and KG (+) phenotype cells by anti-KG (+) sera. KG (+) phenotype cells were agglutinated by anti-KG (+) sera,

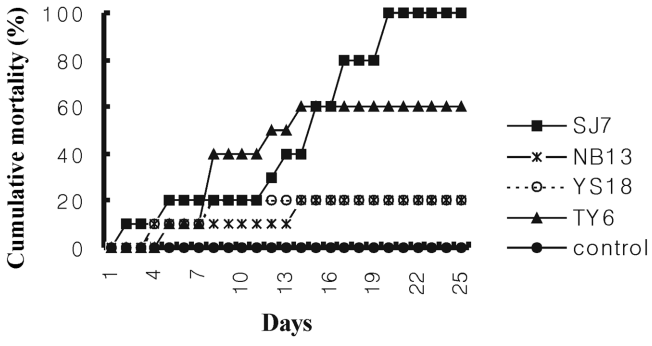

Fig. 1. Comparison of cumulative mortality of rockfish challenged with *L. garvieae* Korean isolates. Fishes were injected intraperitoneally (IP) with *L. garvieae* isolates (1×10^7 CFU $fish^{-1}$). The cumulative mortality was monitored for 25 days at 18°C.

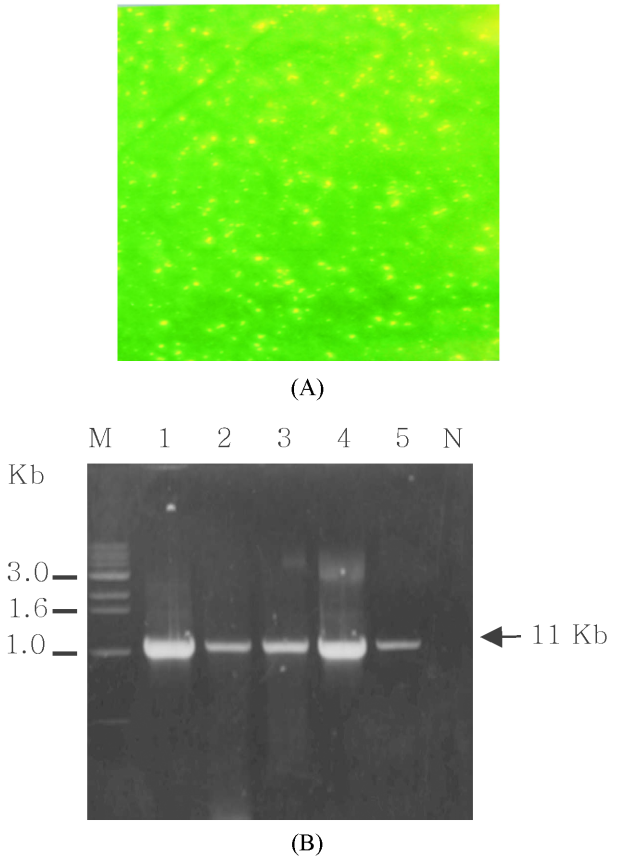

Fig. 2. (A) Identification of causative agents from challenged rockfish with IFAT using chicken IgY raised against *L. garvieae.* (B). PCR results of the isolated bacteria from challenged fishes, lane1: KG (-) 9408 (reference strain); lane 2: SJ7 strain; lane 3: TY6 stain; lane 4: NB13 strain; lane 5: YS18 strain; N: negative control; M: DNA molecular weight marker.

while KG (-) phenotype cells were not agglutinated by anti-KG (+) sera [18,22]. However, both KG (+) and KG (-) phenotype cells were agglutinated when anti-KG (-) sera was introduced [18,19]. According to the agglutination test, both NB13 and TY18 were regarded as KG (+) phenotype cells while both SJ7 and TY6 were thought as KG (-) phenotype cells.

In the challenge studies, after 15 days of post infection fishes exhibited a variety of clinical signs such as abnormal behavior; anorexia, wandering around corner, erect swimming, severe conjunctivitis, melanosis leading mass mortality. There was a significant difference in the mortality of fishes challenged with different bacterial isolates and the control group. Highest mortality was observed from the fishes challenged with isolates SJ7 followed by TY6 and NB13& YS18 at the rate of 100, 60 and 20%, respectively, in 10-20 days of post infection. The IFAT and PCR could detect bacterial pathogen in the internal organs of the fishes challenged with all the four bacterial isolates SJ7, TY6 NB13 and YS18 (Fig. 1-2).

This study clearly showed that the capsular *L. garvieae* isolates are highly pathogenic to black rockfish, and the pathogens are expected to be the causative agents of streptococcosis not only olive flounder but also black rockfish. Moreover, outbreaks of streptococcosis caused by *L. garvieae* in either olive flounder or rock fish might infect nearby fish farms either olive flounder or rock fish.

Acknowledgments

This work was supported by grant No. (R01-2001-000-00242-0) from the Basic Research Program of the Korea Science & Engineering Foundation.

References

1. **Bai SC, Kim KW.** Present status and future prospects of aquaculture in Korea. World Aquacult 2001, **32**, 28-32.
2. **Barnes AC, Guyot C, Hanse BG, Mackenzie K, Horn MT, Ellis AE.** Resistance to serum killing may contribute to differences in the abilities of capsulate and non-capsulated isolates of *Lactococcus garvieae* to cause disease in rainbow trout (*Oncorhynchus mykiss L.).* Fish Shellfish Immunol 2002, **12**, 155-168.
3. **Barnes AC, Young FM, Horne MT, Ellis AE,** *Streptococcus iniae*: serological differences, presence of capsule and resistance to immune serum killing. Dis Aquat Organ 2003, **53**, 241-247.
4. **Carson J, Gudkovs N, Austin B.** Characteristics of an *Enterococcus*-like bacterium from Australia and South Africa, pathogenic for rainbow trout (*Onchorynchus mykiss* Walbaum). J Fish Dis 1993, **16**, 381-388.
5. **Che SC, Lin YD, Liaw LL, Wang PC.** *Lactococcus garvieae* infection in the giant freshwater prawn *Macrobranchium rosenbergii* confirmed by polymerase chain reaction and 16S rDNA sequencing. Dis Aquat Organ 2001, **45**, 45-52.
6. **Collins MD, Ash CJ, Farrow AE, Wallbanks S, Williams AM.** 16S ribosomal ribonucleic acid sequence analyses of lactococci and related taxa. description of *Vagococcus fluvialis* gen. nov., sp. nov. J Appl Bacteriol 1989, **67**, 453-460.
7. **Collins MD, Farrow FAE, Phillips BA, Kandler O.** *Streptococcus garvieae* sp. nov. and *Streptococcus plantarum* sp. nov. J Gen Microbiol 1984, **129**, 3427-3431.
8. **Domenech A, Prieta J, Fernandez-Garayzabal JF, Collins MD, Jones D, Dominguez L.** Phenotypic and phylogenetic evidence for a close relationship between *Lactococcus garvieae* and *Enterococcus seriolicida*. Microbiologia 1993, **9**, 63-68.
9. **Eldar A, Ghittino C, Asanta L, Bozzetta E, Goria M, Prearo M, Bercovier H.** *Enterococcus seriolicida* is a junior synonym of *L. garvieae*, a causative agent of septicemia and meningoencephalitis in fish. Curr Microbiol 1996, **32**, 85-88.
10. **Eldar A, Hurvitz A, Bercovier H, Ghittino C.** *Lactococcus garvieae* and *Streptococcus iniae* infections in rainbow trout (*Oncorhynchus mykiss*): two similar but different diseases.

Dis Aquat Organ 1999, **36**, 227-231.
11. **Elliott JA, Collins MD, Pigott NE, Facklam RR.** Differentiation of *Lactococcus lactis* and *Lactococcus garvieae* from humans by comparison of whole-cell protein patterns. J Clin Microbiol 1991, **29**, 2731-2734.
12. **Fefer JJ, Ratzan KR, Sharp SE, Saiz E.** *Lactococcus garvieae* endocarditis: report of a case and review of the literature. Diagn Microbiol Infect Dis 1998, 127-130.
13. **James PR, Hardman SMC, Patterson DLH.** Osteomyelitis and possible endocarditis secondary to *Lactococcus garvieae*: a first case report. J Postgrad Med J 2000, **76**, 301-303.
14. **Kim IB.** Cage Aquaculture in Korea. Proceedings of the first International Symposium on Cage Aquaculture in Asia. In: Liao IC, Lin CK (eds.), Cage Aquaculture in Asia 2000, pp. 59-73, World Aquaculture Society, Charleston, 2000.
15. **Kusuda R, Kawai K, Salati F, Banner CR, Freyer JL.** *Enterococcus seriolicida* sp. nov., a fish pathogen. Int J Syst Bacteriol 1991, **41**, 406-409.
16. **Lee S, Jeon IG, Lee JY.** Effects of digestible protein and lipid levels in practical diets on growth, protein utilization and body composition of juvenile Rockfish (*Sebastes schlegeli*). Aquaculture 2002, **211**, 227-239.
17. **Lee SB, Mine Y, Stevenson RM.** Effects of hen egg yolk immunoglobulin in passive protection of rainbow trout against *Yersinia ruckeri*. J Agric Food Chem 2000, **48**, 110-115.
18. **Ooyama T, Hirokawa Y, Minami T, Yasuda H, Nakai T, Endo M, Ruangan L, Yoshida T.** Cell-surface properties of *Lactococcus garvieae* strains and their immunogenicity in the yellowtail (*Seriola quinqueradiata*). Dis Aquat Organ 2002, **51**, 169-177.
19. **Ooyama T, Kera A, Okada T, Inglis V, Yoshida T.** The protective immune response of yellowtail (*Seriola quinqueradiata*) to the bacterial fish pathogen *Lactococcus garvieae*. Dis Aquat Organ 1999, **37**, 121-126.
20. **Schmidtke LM, Carson J.** Induction, characterisation and pathogenicity in rainbow trout *Oncorhynchus mykiss* (Walbaum) of *Lactococcus garvieae* L-forms. Vet Microbiol 1999, **69**, 287-300.
21. **Teixeira LM, Merquior VLC, Vianni MCE, Carvalho MGS, Fracalanzza SEL, Steigerwalt AG, Brenner DJ, Facklam RR.** Phenotypic and genotypic characterization of atypical *Lactococcus garvieae* strains isolated from water buffalos with subclinical mastitis and confirmation of *L. garvieae* as a senior subjective synonym of *Enterococcus seriolicida*. Int J Syst Bacteriol 1996, **46**, 664-668.
22. **Yoshida T, Endo M, Sakai M, Inglis V.** A cell capsule with possible involvement in resistance to opsonophagocytosis in *Enterococcus seriolicida* isolated from yellowtail *Seriola quinqueradiata*. Dis Aquat Organ 1997, **29**, 233-235.
23. **Zlotkin A, Eldar A, Ghittino C, Bercovier H.** Identification of *Lactococcus garvieae* by PCR. J Clin Microbiol 1998, **36**, 983-985.