Zand Molecular Microbiology
Volume 1 Number 2 (December 2025) 88 - 97
DOI: https://doi.org/10.22034/zmm.2025.736067

Original Article

Molecular surveillance and pathogenicity assessment of *Lactococcus garvieae* and *Streptococcus iniae* in Rainbow trout

Ali Shirazinezhad [1], **Hossein Khaj** [2], **Seyyed Mohammad Hossein Hosseini** [1], **Fatemeh Dabiri** [1]
Hajar Molaee [1], **Yahya Tahamtan** [1], **Masoumeh Hayati** [1]

[1] Department of Microbiology, Shiraz Branch, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Shiraz, Iran.
[2] Department of Immunology, Shiraz Branch, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Shiraz, Iran.

Received: *October 2025,* ***Accepted:*** *November 2025*

Abstract

Background & Objectives: Streptococcosis, primarily caused by *Streptococcus iniae* and *Lactococcus garvieae*, causes high mortality and economic losses in rainbow trout (*Oncorhynchus mykiss*) worldwide. The disease is systemic, associated with septicemia and hemorrhages, and vaccination is the most effective preventive strategy. Since successful vaccination depends on highly virulent strains, this study isolated and characterized streptococcal pathogens from diseased fish to select a potent vaccine candidate.
Materials & methods: In this experimental study, 111 clinical samples were collected from rainbow trout with clinical signs of streptococcosis from commercial fish farms. Identification was conducted via experimental culture methods, biochemical profiling and 16S rRNA gene PCR were used for bacterial characterization. In vivo virulence was assessed by determining the median lethal dose (LD_{50}) in rainbow trout.
Results: Bacteriological analysis of clinical samples revealed that 95% of isolates were gram-positive cocci by staining. Molecular techniques confirmed 37.84% of isolates as *Lactococcus garvieae* (1100 bp product) and 18.92% as *Streptococcus iniae* (300 bp product), while 43.24% of isolates yielded no identifiable *L. garvieae* or *S. iniae*. Virulence assessment via the Reed and Muench method revealed LD_{50} values of $10^{2.41}$ for *L. garvieae* isolate LG001 and $10^{2.83}$ for *S. iniae* isolate SE001, indicating high pathogenicity in both isolates.
Conclusion: This study provides essential data for the selection of a highly virulent streptococcal strain as a vaccine candidate and offers a scientific basis for developing an effective vaccine to control streptococcosis in rainbow trout, thereby reducing economic losses in aquaculture and minimizing zoonotic risks.

Keywords: *Lactococcus garvieae;* LD_{50}; Rainbow trout; *Streptococcus iniae.*

Corresponding author: **Ali Shirazinezhad, PhD., Department of Microbiology, Shiraz Branch, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Shiraz, Iran.**
Tel: +98-9177180252 E-mail: shirazin2002@yahoo.com

Introduction

Aquaculture has become one of the fastest growing food production sectors worldwide, playing a vital role in global food security by providing an essential source of high-quality protein for millions of people (1). However, the rapid expansion of fish farming has been accompanied by a rise in infectious diseases, particularly those caused by bacterial pathogens, which compromise fish health, reduce productivity, and can lead to substantial economic losses in fish farms (2). One of the most important bacterial diseases in aquaculture is streptococcosis, a septicemic infection that causes severe economic damage in both marine and freshwater fish species across the globe (3). Streptococcosis outbreaks are often temperature-dependent, typically occurring in warmer waters at temperatures above 15 °C, while cold-water outbreaks are less frequent and occur below this threshold (4). The primary causative agents in this infection are *Lactococcus garvieae* and *Streptococcus iniae*, both of which are Gram-positive cocci known to induce severe systemic infections in fish (5,6). *L. garvieae* was formerly classified as a member of the lactic acid bacteria, but was later reclassified as based on genetic and phenotypic characteristics (7). It predominantly affects warm-water species such as yellowtail and trout, causing hemorrhaging and multi-organ failure (8). In contrast, *S. iniae* is a versatile pathogen capable of infecting a broad range of fish species, including tilapia and groupers, particularly in tropical and subtropical regions (6). Outbreaks associated with these pathogens typically occur at temperatures between 15–20 °C (9).

L. garvieae was first reported as a disease in an intensive rainbow trout (*Oncorhynchus mykiss*) farm in Japan and also has since been identified in numerous human clinical cases that specify its potential role as a zoonotic pathogen (10). While *S. iniae* is familiar for its impact on aquaculture, it has also gained attention as a zoonotic risk and causing infections in humans through injuries from handling infected fish (11). *S. iniae* infection in trout causes more chronic progression obvious by distinct lesions, whereas *L. garvieae* leads to acute systemic disease and rapid mortality (8).

Accurate identification and characterization of these pathogens are essential for the development of effective control measures, including targeted antibiotic treatments, vaccination strategies, and biosecurity protocols. Traditional bacteriological methods, while useful, may be insufficient for distinguishing closely related strains. Hence, molecular techniques such as polymerase chain reaction (PCR) is increasingly employed to complement phenotypic analyses and ensure precise identification.

Besides the identification, understanding the pathogenic potential of these bacteria through in vivo studies provides critical insight into their virulence mechanisms. This information is vital for evaluating the risk they pose under farm conditions and for designing effective preventive strategies.

Due to the limited information regarding the development of effective fish vaccines in Iran, and a need for isolation of *L. garvieae* and *S. iniae*, this study aimed to isolate and identify *L. garvieae* and *S. iniae* from diseased fish from different area of Iran, characterize them using biochemical and molecular methods, and evaluate their pathogenicity under experimental conditions. The findings are predictable to enhance the understanding of these pathogens biology and epidemiology and to support the development of more effective disease management strategies in aquaculture.

Materials and Methods

A) Sampling: In this experimental study, a total of 111 individual samples of fishes were collected from different aquaculture farms across various regions of Iran, including 80 specimens from diseased rainbow trout exhibiting clinical signs of streptococcosis and 31 bacterial isolates obtained from suspected fish. Sampling was conducted at aquaculture farms situated in Bushehr, Chaharmahal and Bakhtiari, Khuzestan, Fars, Kohgiluyeh and Boyer-Ahmad, and Mazandaran. The specimens were transported under sterile conditions to the Microbiology Laboratory of the Razi Vaccine and Serum Research Institute, Shiraz branch. After recording of clinical signs, internal organs including kidney, liver, and brain were aseptically dissected and inoculated onto various culture media like Blood Agar (BA), Tryptic Soy Broth (TSB), Tryptic Soy Agar (TSA), and Brain Heart Infusion (BHI) media and incubated at 37°C .

B) Biochemical properties: Grayish-white, catalase and oxidase negative pure colonies were subjected to Gram staining. Then, the biochemical characterization of the isolates was carried out according to the protocol recommended by different researches for *L. garvieae* and *S. iniae* (12). All suspected *L. garvieae* and *S. iniae* isolates were assessed for bacterial growth potentials at 37 °C, and tests including catalase, oxidase, MR-VP (Voges-Proskauer) reactions, hydrolysis, different sugar consumption, oxidation and fermentation of glucose (O/F), production of indole and H_2S, and SIM (sulfide-indole-motility), Merck, Darmstadt, Germany) medium. All of these tests were read after 24 h of incubation at 37 °C. In addition, the growth potential of the isolates was tested to evaluate the salt tolerance, using three variations of BHI medium including: an untreated control (BHI alone), and two others supplemented with sodium chloride (NaCl) at concentrations of 5% and 6.5%. Briefly, 10 µL of bacterial suspension in sterile PBS (phosphate-buffered saline) was aseptically inoculated in duplicate different BHI broth. The inoculated tubes were incubated at 37°C for 24 hours, after which bacterial growth was evaluated.

C) Molecular Identification:

1. DNA Isolation: Suspected bacterial isolates were subjected to DNA extraction. A single colony of each isolate was suspended in 100 µL of sterile distilled water. The suspension was then heated at 95 °C for 10 minutes in a TermoBlok to lyse the bacterial cells and release genomic DNA (13). After boiling, the tubes were centrifuged at 12,000 g for 2 minutes to pellet cellular debris. The supernatant containing crude DNA was carefully transferred to a new sterile tube and stored at -20 °C for downstream molecular analyses.

2. PCR assay: Definitive identification of *L. garvieae* and *S. iniae* was done by polymerase chain reaction assay targeting the 16S rRNA gene (14). Species-specific primers were utilized to amplify an approximately 1100 bp fragment for *L. garvieae* and a 300 bp fragment for *S. iniae*, in accordance with previously established protocols (15). The Sequences of the primers are listed in Table 1.

3. Pathogenicity of isolates in rainbow trout: To determine bacterial severity, the conventional LD50 method was employed using the Reed and Muench technique (16). Briefly, bacteria in the logarithmic phase were harvested by centrifugation at 4500 g for 10 minutes, washed with sterile PBS, and diluted tenfold from 10^2 to 10^7 CFU/ml, confirmed by McFarland standards and viable counts. For each isolate, 210 rainbow trout (25 g) were allocated

Table 1. Primer sequences of isolates.

Target Gene	Organism	Amplicon Size	Primer Name	Sequence (5′–3′)	Reference
16S rRNA	*S. iniae*	300 bp	Sin1 (forward)	CTAGAGTACACATGTAGCTAAG	(15)
			Sin2 (reverse)	GGATTTTCCACTCCCATTAC	
16S rRNA	*L. garvieae*	1100 bp	Lg1 (forward)	CATAACAATGAGAATCGC	(15)
			Lg2 (reverse)	GCACCCTCGCGGGTTG	

into six groups of 35 fish. Following anesthesia with clove extract, fishes were intraperitoneally injected with 0.1 ml of bacterial suspension corresponding to the designated protocol. Controls included a group injected with sterile PBS (negative control) and an uninjected handling control. Mortality was recorded daily over 10 days. To confirm the cause of death, bacterial cultures were performed on the kidneys and brains of deceased fish, with successful re-isolation of the injected bacteria. Finally, LD50 calculated with the Reed-Muench LD_{50} calculation formula:

$$LD50 = \log_{10}(lower\ dilution) + \left(\frac{50 - \%\ mortality\ below}{\%\ mortality\ above - \%\ mortality\ below}\right) \times \log_{10}(dilution\ factor)$$

In this formula, the lower dilution is the dilution just below 50% mortality; percentage mortality above refers to the mortality at the next higher (more concentrated) dilution exceeding 50%; percentage mortality below is the mortality at the next lower (more diluted) dilution below 50%; and the dilution factor is the fold difference between serial dilutions, which equals 1 when using logarithmic values in a tenfold dilution series .

Results

A) Isolated samples: Out of 111 samples collected and cultured on selective and differential media, isolates were subjected to gram staining following incubation, which revealed that approximately 95% were gram-positive cocci. Species-level identification by using PCR and species-specific primers, confirmed 42 isolates (37.84%) as *L. garvieae* and 21 isolates (18.92%) as *S. iniae.* Approximately 43.24% of the received specimens remained uncharacterized, as none of the identified bacteria matched those samples. Comprehensive data on the isolation of *S. iniae* and *L. garvieae* by province are presented in Table 2.

Table 2. Distribution of bacterial isolates by geographic origin.

		Isolated samples	
Province	**Received samples**	***L. garvieae* (%)**	***S. iniae* (%)**
Bushehr	18	6 (5.41%)	4 (3.60%)
Chaharmahal and Bakhtiari	15	6 (5.41%)	3 (2.70%)
Khuzestan	12	5 (4.50%)	2 (1.80%)
Fars	37	13 (11.71%)	6 (5.41%)
Kohgiluyeh and Boyer-Ahmad	20	8 (7.21%)	4 (3.60%)
Mazandaran	9	4 (3.60%)	2 (1.81%)
Total	111 (100%)	42 (37.84%)	21 (18.92%)

B) Biochemical test: In the biochemical tests, both species showed negative results for indole, citrate, motility, catalase, and oxidase, with MR/VP positive/negative and Alk/A on TSI. NaCl tolerance differentiated them: *L. garvieae* grew at 5% and 6.5% NaCl, whereas *S. iniae* did not.

C) Molecular Identification: Molecular techniques were applied to verify the identities of isolates initially suspected based on staining and biochemical assays. PCR amplification confirmed that 37.84% of the isolates corresponded to *L. garvieae*, as evidenced by a distinct 1100 base pair band (Figure 1), and 18.92% of the isolates were identified as *S. iniae*, indicated by a specific 300 base pair PCR product (Figure 2).

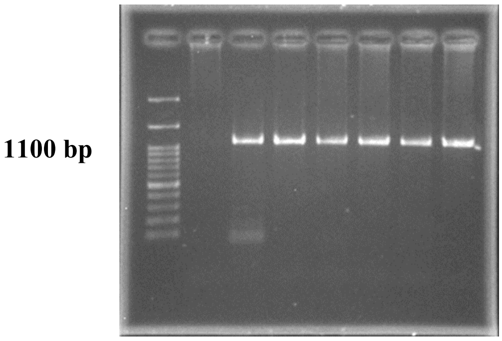

Fig 1. Gel electrophoresis of PCR products targeting the *L. garvieae* (1100 bp). From left to right: Lane 1 contains a 100 bp DNA ladder (M), followed by the negative control (C^-), positive control (C^+), and test samples (1-5).

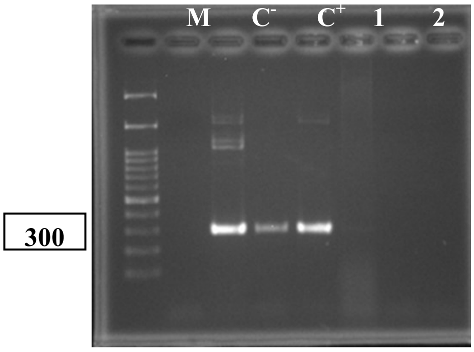

Fig 2. Gel electrophoresis of PCR products targeting the *S. iniae* (300 bp). From left to right: Lane 1 contains a 100 bp DNA ladder (M), followed by the negative control (C^-), positive control (C^+), and test samples (1-3).

D) LD50 results: Given the similarity of the isolates in both biochemical and molecular characteristics, all *L. garvieae* and *S. iniae* isolates were subjected to LD50 testing to assess their virulence (Table 3). The results of the LD50 analysis are presented in Table 3 for all *L. garvieae* and *S. iniae* isolates.

Table 3. LD50 obtained by Reed and Muench method.

	L. garvieae	**LD50**	***S. iniae***	**LD50**
1	LG001	$10^{2.41}$	SE001	$10^{2.83}$
2	LG002	$10^{4.21}$	SE002	$10^{4.23}$
3	LG003	$10^{3.88}$	SE003	$10^{4.13}$
4	LG004	$10^{2.63}$	SE004	$10^{3.25}$
5	LG005	$10^{4.76}$	SE005	$10^{3.64}$
6	LG006	$10^{2.81}$	SE006	$10^{4.28}$
7	LG007	$10^{3.42}$	SE007	$10^{3.82}$
8	LG008	$10^{4.73}$	SE008	$10^{3.21}$
9	LG009	$10^{4.97}$	SE009	$10^{4.47}$
10	LG010	$10^{4.06}$	SE010	$10^{4.91}$
11	LG011	$10^{5.12}$	SE011	$10^{3.54}$
12	LG012	$10^{3.49}$	SE012	$10^{2.96}$
13	LG013	$10^{3.17}$	SE013	$10^{3.31}$
14	LG014	$10^{3.98}$	SE014	$10^{3.14}$
15	LG015	$10^{4.18}$	SE015	$10^{3.97}$
16	LG016	$10^{3.79}$	SE016	$10^{2.89}$
17	LG017	$10^{2.96}$	SE017	$10^{3.75}$
18	LG018	$10^{3.65}$	SE018	$10^{3.45}$
19	LG019	$10^{4.35}$	SE019	$10^{4.02}$
20	LG020	$10^{4.27}$	SE020	$10^{4.35}$
21	LG021	$10^{3.13}$	SE021	$10^{2.92}$
22	LG022	$10^{3.38}$		
23	LG023	$10^{3.55}$		
24	LG024	$10^{2.86}$		
25	LG025	$10^{5.15}$		
26	LG026	$10^{4.73}$		
27	LG027	$10^{4.96}$		
28	LG028	$10^{5.05}$		
29	LG029	$10^{4.78}$		
30	LG030	$10^{2.91}$		
31	LG031	$10^{3.72}$		
32	LG032	$10^{3.84}$		
33	LG033	$10^{4.27}$		
34	LG034	$10^{4.31}$		
35	LG035	$10^{4.62}$		
36	LG036	$10^{5.01}$		
37	LG037	$10^{2.88}$		
38	LG038	$10^{3.15}$		
39	LG039	$10^{4.51}$		
40	LG040	$10^{2.94}$		
41	LG041	$10^{3.87}$		
42	LG042	$10^{5.02}$		

The LD50 values were determined using Probit analysis. Among the isolated bacterial strains, the one with the lowest LD50 was selected, with its details presented in Table 4.

Table 4. Ten-day LD_{50} data for *L. garvieae* (LG001) and *S. iniae* (SE001) isolates in rainbow trout based on serial dilution mortality rates.

		***L. garvieae* isolate LG001**		***S. iniae* isolate SE001**	
Dilution	***Fish Tested***	***Fish Dead***	***Mortality rate %***	***Fish Dead***	***Mortality rate %***
10^7	25	25	100%	25	100%
10^6	25	25	100%	25	100%
10^5	25	25	100%	25	100%
10^4	25	20	80%	20	80%
10^3	25	16	64%	14	56%
10^2	25	10	40%	5	20%

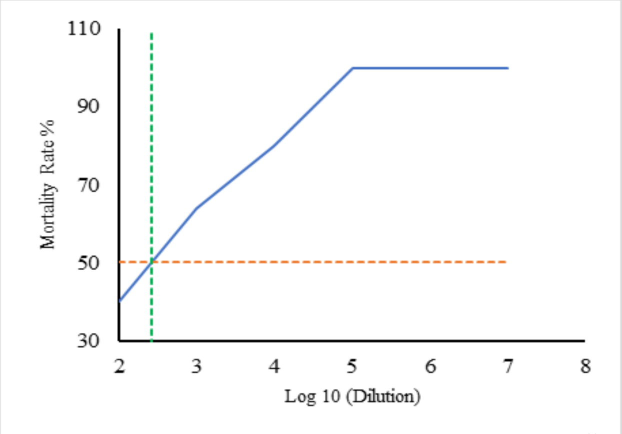

Fig 3. Dose-response curve of L. garvieae LG001. Mortality rate (%) vs. $\log_{10}$(dilution). Horizontal dashed line: 50% mortality. Vertical dashed line from curve to x-axis indicates LD_{50} dilution.

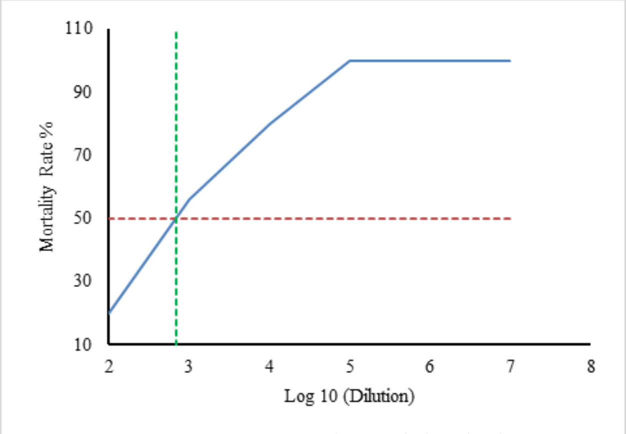

Fig 4. Dose response curve for S. iniae isolate SE001. The horizontal and vertical dashed lines intersect on the curve at the point corresponding to 50% mortality (LD_{50}), and the vertical line marks the LD_{50} dilution on the x-axis.

The dose response relationship, expressed as the percentage mortality against the logarithm (Log_{10}) of dilution factor, is illustrated in Figure 3 for *L. garvieae* isolate LG001 and Figure 4 for *S. iniae* isolate SE001. The point where the horizontal dashed line (50% mortality) meets the dose-response curve, and from which the vertical dashed line drops to the x-axis, represents the median lethal dose (LD_{50}). This intersection provides a graphical estimation of the dilution required to kill 50% of the host population. According to the Reed and Muench method, the estimated LD50 values were $10^{2.41}$ for *L. garvieae* isolate LG001 and $10^{2.83}$ for *S. iniae* isolate SE001.

Discussion

Infectious diseases represent a critical constraint to the sustainable growth and economic viability of the global aquaculture industry. The Iranian aquaculture sector similarly faces significant challenges, as evidenced by several studies conducted by local researchers (17,18). Among the various disease management strategies, vaccination remains the most cost-effective, practical, and environmentally sound approach for the prevention and control of infectious diseases in aquatic species. In Iran, researches aimed at the development and optimization of aquatic vaccines have been initiated and are progressively advancing within specialized research institutions (19). Within this framework, RVSRI-recognized as the principal authority in the research, development, and production of livestock and poultry vaccines in Iran-has initiated a strategic program aimed at the isolation, molecular characterization, and virulence profiling of *L. garvieae* and *S. iniae* strains from clinical cases of streptococcosis in rainbow trout aquaculture systems. The primary objective is the identification and selection of highly virulent strains for subsequent vaccine formulation. In the present study, clinical specimens and bacterial isolates from diseased rainbow trout suspected of streptococcal infection were systematically collected, resulting in the acquisition of 42 *L. garvieae* and 21 *S. iniae* isolates. The inability to recover viable cultures from certain specimens is likely attributable to factors such as suboptimal bacterial regrowth during sampling or cultivation, prior administration of antimicrobial agents, or bacterial degradation during transport, all of which may compromise bacterial viability and isolation success.

89% of the samples cultured on blood agar and TSA, exhibited colonies with streptococcal characteristics, while the rest colonies lacked these features and were likely associated with other pathogenic or non-pathogenic fish bacteria. Smears were prepared from colonies grown on blood agar or TSA, followed by Gram staining. Among the colonies with streptococcal characteristics, more than 60% of the samples revealed Gram-positive cocci arranged in long chains, suggesting the presence of *L. garvieae*, consistent with previous reports indicating that this species typically forms relatively long chains (20). In the other cases, gram staining revealed gram-positive cocci predominantly in pairs or short chains, which, based on the literatures, are characterized as *S. iniae* (21). Biochemical testing of the Gram-positive cocci isolates revealed that they were negative in all assays except for growth in NaCl-enriched media. Notably, 50% of these isolates grew in media containing 5% and 6.5% NaCl, a growth profile consistent with *L. garvieae*, which is known to tolerate up to 6.5% NaCl (22). Conversely, 27% of the isolates failed to grow in salt-supplemented media, aligning with the recognized salt intolerance of *S. iniae* (23).

In the present study, bacterial isolates initially identified through gram staining and biochemical tests, 42 isolates were confirmed as *L. garvieae* by the amplification of a 1100 base pair fragment, while the remaining 21 isolates were confirmed as *S. iniae* by the amplification of a 300 base pair fragment, as previously described (24). Previous studies on the prevalence of *L. garvieae* in rainbow trout farms have demonstrated the widespread occurrence of infections caused by this pathogen in aquaculture systems in Iran (15). After confirming the isolates, their pathogenicity was evaluated to determine the most virulent isolates. This was accomplished by assessing the LD_{50} of each isolate using the Reed and Muench method (25). Finally, the isolates exhibiting the highest virulence, with a LD_{50} of $10^{2.41}$ for *L. garvieae* and $10^{2.83}$ for *S. iniae*, were selected for vaccine production and preserved at −70°C.

Conclusion

Streptococcosis caused by *Streptococcus iniae* and *Lactococcus garvieae* poses a significant threat to rainbow trout aquaculture, resulting in high mortality, economic losses, and potential zoonotic risks. In this study, clinical isolates from diseased trout were successfully identified through biochemical and molecular methods, and their virulence was evaluated using LD_{50} assays. The most virulent isolate was identified and preserved as a promising candidate for future vaccine development. These findings provide a solid foundation for targeted vaccination strategies aimed at controlling streptococcosis, improving fish health, and reducing both economic and public health impacts.

Acknowledgment

The authors would like to express their sincere gratitude to the Razi Vaccine and Serum Research Institute, Shiraz Branch, for their valuable support throughout this study. Special thanks are extended to the technical staff for their assistance with PCR procedures and laboratory analyses. Also, authors thank Mr. S. Sadeghzadeh (Laboratory Animal Department) for his technical assistance.

Conflicts of interest

The authors state that there are no conflicts of interest regarding this publication.

Funding

This study was conducted as part of research project No. (0-84-18-056-000914) approved by the Razi Vaccine and Serum Research Institute.

References

1. Mood A LE, Boyland NK, Brooke P. Estimating global numbers of farmed fishes killed for food annually from 1990 to 2019: Animal Welfare; 2023.
2. Bondad-Reantaso MG, Subasinghe RP, Arthur JR, Ogawa K, Chinabut S, Adlard R, Tan Z, Shariff M. Disease and health management in Asian aquaculture. Veterinary parasitology. 2005;132(3-4):249-72.
3. Pastorino P, Vela Alonso AI, Colussi S, Cavazza G, Menconi V, Mugetti D, Righetti M, Barbero R, Zuccaro G, Fernández-Garayzábal JF. A summer mortality outbreak of Lactococcosis by *Lactococcus garvieae* in a raceway system affecting farmed rainbow trout (Oncorhynchus mykiss) and brook trout (Salvelinus fontinalis). Animals. 2019;9(12):1043.
4. Amal M, Zamri-Saad M. Streptococcosis in tilapia (Oreochromis niloticus): a review. Pertanika Journal of Tropical Agricultural Science. 2011;34(2):195-206.
5. Mileriene J, Serniene L, Kasparaviciene B, Lauciene L, Kasetiene N, Zakariene G, Kersiene M, Leskauskaite D, Viskelis J, Kourkoutas Y. Exploring the potential of sustainable acid whey cheese supplemented with apple pomace and GABA-producing indigenous *Lactococcus lactis* strain. Microorganisms. 2023;11 (2):436.
6. Juárez-Cortés MZ, Vázquez LEC, Díaz SFM, Cardona Félix CS. *Streptococcus iniae* in aquaculture: A review of pathogenesis, virulence, and antibiotic resistance. International Journal of Veterinary Science and Medicine. 2024;12(1):25-38.
7. Patel P, Patel B, Amaresan N, Joshi B, Shah R, Krishnamurthy R. Isolation and characterization of *Lactococcus garvieae* from the fish gut for in vitro fermentation with carbohydrates from agro-industrial waste. Biotechnology Reports. 2020;28:e00555.
8. Vendrell D, Balcázar JL, Ruiz-Zarzuela I, De Blas I, Gironés O, Múzquiz JL. *Lactococcus garvieae* in fish: a review. Comparative immunology, microbiology and infectious diseases. 2006;29(4):177-98.
9. Rodkhum C, Kayansamruaj P, Pirarat N. Effect of water temperature on susceptibility to *Streptococcus agalactiae* serotype Ia infection in Nile tilapia (Oreochromis niloticus). The Thai Journal of Veterinary Medicine. 2011;41(3):309-14.
10. Hoshina T. A Streptococcus pathogenic to fish. J Tokyo Univ Fish. 1958;44:57-68.
11. Al-Harbi AH. Molecular characterization of *Streptococcus iniae* isolated from hybrid tilapia (Oreochromis niloticus× Oreochromis aureus). Aquaculture. 2011;312(1-4):15-8.
12. Austin B, Austin DA. Bacterial fish pathogens. 2 E, editor. Heriot-Watt University, Edinburgh, UK: Aquaculture and Fisheries Support Series; 1993.
13. Molaee H, Tahamtan Y, Saeednezhad E, Hayati M. Isolation of the various serotypes of *Mannheimia haemolytica* and preparation of the first vaccine candidate in Iran. Molecular Biology Reports. 2022;49(11):10367-75.
14. Sirakov I, Strateva TV, Boyanov VS, Orozova P, Yordanov D, Rusenova N, Gergova R, Dimov SG, Sirakova B, Radosavljević V. Identification, Characterization, and Epidemiological Analysis of *Lactococcus garvieae* Fish Isolates Obtained in a Period of Eighteen Years. Microorganisms. 2025;13 (2):436.
15. Fadaeifard F, Momtaz H, Rahimi E, Mirzakhani A. Detection of *Streptococcus iniae* and *Lactococcus garvieae* by multiplex polymerase chain reaction (PCR) in some rainbow trout farms of Iran. African Journal of Biotechnology. 2012;11(2):260-3.
16. Reed LJ, Muench H. A simple method of estimating fifty per cent endpoints. 1938.
17. Rahmatullah M, Ariff M, Kahieshesfandiari M, Daud H, Zamri-Saad M, Sabri M, Amal M, Ina-Salwany M. Isolation and pathogenicity of *Streptococcus iniae* in cultured red hybrid tilapia in Malaysia. Journal of Aquatic Animal Health. 2017;29(4):208-13.
18. Daneshamouz S, Haghi F, Zeighami H. Detection and identification of bacterial pathogens in rainbow trout (Oncorhynchus mykiss) samples from fish farms in Iran. Thalassas: An International Journal of Marine Sciences. 2020;36:133-41.
19. Hosseini MH, Akhlaghi M, Moazzeni Jula G. Experimental vaccine against lactococcosis in cultured rainbowtrout (Oncorhynchus mykiss). Archives of Razi Institute. 2011;66(1):51-7.

20. Gibello A, Galán-Sánchez F, Blanco MM, Rodríguez -Iglesias M, Domínguez L, Fernández-Garayzábal JF. The zoonotic potential of *Lactococcus garvieae*: An overview on microbiology, epidemiology, virulence factors and relationship with its presence in foods. Research in veterinary science. 2016;109: 59-70.
21. Milani CJ, Aziz RK, Locke JB, Dahesh S, Nizet V, Buchanan JT. The novel polysaccharide deacetylase homologue Pdi contributes to virulence of the aquatic pathogen *Streptococcus iniae*. Microbiology. 2010;156(2):543-54.
22. Navas M, Hall G, El Bejjani D. *Lactococcus garvieae*: a case report and suggested methods for identification. Journal of Clinical Microbiology. 2013.
23. Numberger D, Siebert U, Fulde M, Valentin-Weigand P. Streptococcal infections in marine mammals. Microorganisms. 2021;9(2):350.
24. Farzadnia A, Naeemipour M. Molecular techniques for the detection of bacterial zoonotic pathogens in fish and humans. Aquaculture International. 2020;28:309-20.
25. Saganuwan SA. Application of median lethal concentration (LC 50) of pathogenic microorganisms and their antigens in vaccine development. BMC Research Notes. 2020;13:1-6.