

Original Article

Long-time Immune Response of Rainbow Trout (*Oncorhynchus mykiss*) to a Local Yersiniosis Vaccine (Antiyersin) Under Farm Conditions



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ABSTRACT

Background: Yersiniosis caused by *Yersinia ruckeri* is an economically important disease in aquaculture, especially rainbow trout farms. Developing an effective vaccination to combat this disease is an important strategy for improving fish production.

Objectives: This study aimed to determine long-term immune response of rainbow trout to Antiyersin vaccine under field conditions.

Methods: Two vaccination routs, immersion (1:9 V/V) and intraperitoneal injection (0.2 ml/fish) were used. Antibody titer was recorded by enzyme-linked immunosorbent assay (ELISA) and agglutination plus immunocompetent cell population.

Results: No significant difference in heterophil value was observed between single immersion vaccination (SIV) and immersion vaccination plus booster (IVB) two months post-vaccination and between IBV and intraperitoneal vaccination (IPV) three and four months post-vaccination. Generally, the highest lymphocyte value was recorded in IPV, followed by IVB and SIV, respectively. Also, in general, there were no significant differences in monocyte value among the treatments during the study. IPV and IVB demonstrated significantly higher antibody titers than SIV, as measured by agglutination assay at all studied time points. The antibody titers by ELISA in all vaccinated groups were significantly higher than those in control fish. No significant difference was observed between IVB and IPV two months post-study, but both groups exhibited significantly higher levels than SIV.

Conclusion: These data are useful for developing national policies to improve disease management in trout aquaculture. The results of the present study showed that the IVB route can be recommended for practical aquaculture because of its effective immune response and its ease of use.

Keywords: Agglutination, ELISA, Leukocyte, Vaccine, *Yersinia ruckeri*

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Introduction

Many microbial pathogens have affected the health of aquatic animals, and subsequently human public health; bacterial diseases have particular importance (Duman et al., 2023). Yersiniosis is one of the most important bacterial diseases in farmed fish, causing significant economic losses to the aquaculture industry of Iran (Soltani et al., 2014a; Soltani et al., 2016; Pennisi, 2020). In Iran, most fish farmers are currently using various antibiotics frequently to reduce the morbidity and mortality. Such frequent treatments have raised antibiotic resistance (Soltani et al., 2023). Yersiniosis, also known as red mouth disease, is a systemic disease that was initially identified in several rainbow trout farms in the United States (Pajdak-Czaus et al., 2019) and has since been observed on all continents of the world (Ummey et al., 2021). *Yersinia ruckeri* from the Enterobacteriaceae family and the causative agent of yersiniosis is a gram-negative, rod-shaped, non-spore-forming, motile bacterium with peritrichous flagella (although non-motile strains also exist) (Lang et al., 2013). Two biotypes of this bacterium have been identified to date, with the majority of pathogenic cases attributed to biotype I (Guijarro et al., 2018; Fernández et al., 2007). This disease has been observed in several commercially important fish species, but rainbow trout are the most susceptible among aquatic animals to this disease (Wang et al., 2021).

Various methods have been proposed for the control and prevention of the disease, with vaccination being recommended as the best method (Soltani et al., 2016; Shafiei et al., 2018; Raida et al., 2011). Since the first commercial vaccine against yersiniosis in 1976, several local vaccines have been introduced to aquaculture industry, including farmed rainbow trout in Iran (Soltani et al., 2016; Shafiei et al., 2018). Given the economic importance and annual losses to the aquaculture industry, as well as the technical knowledge required for vaccine development, it seems essential to determine the humoral immune response induced by vaccination in rainbow trout under farm conditions over a long period.

Materials and Methods

Experimental design

This study was conducted in a rainbow trout farm. The rainbow trout had a mean weight of 70 g (70±5 g) and no history of disease. Health assessments were performed using health-confirmation tests, including the

determination of antibody concentration to yersiniosis in small ponds containing a minimum of 1200 trout. Other cultural conditions were similar to those in typical farm conditions. The fish were kept for three weeks for health monitoring and to acclimate to the training environment before the experiment started. The fish were fed according to water temperature and growth rate using commercial plates produced by Faradaneh Aquatic Animal Feed Company. All animal experiments were performed under the ARRIVE guidelines for the use of animals in research, approved by the University of Tehran (code number: 00112), and following the regulations of national and international institutions.

Water quality

Cultural conditions and water quality are very important in conducting such experiments. For this reason, water quality factors, including temperature, pH, dissolved oxygen, and toxic gases (ammonia and nitrite) were measured and recorded regularly throughout the study. All water quality factors were maintained within safe and acceptable ranges for the health and growth of rainbow trout during the study period.

Vaccination

The yersiniosis vaccine (Antiyersin) was purchased from Boujan Tak Pharmed Company, and was made from killed local strains of *Y. ruckeri* isolated from rainbow trout. The experimental treatments are given in Table 1. Fish in the control tank did not receive any vaccine (group 1). Vaccination was performed using the single immersion method, according to the manufacturer's instructions (1:9 v/v for 2 min) for fish in two ponds simultaneously (groups 2 and 3). Fish in group 3 received a booster dose of the vaccine after 45 days, and fish in group 4 received an intraperitoneal injection of vaccine (0.2 mL/ fish).

Hyperimmune serum preparation

Hyperimmune serum preparation was performed as follows: 15 healthy fish weighing 300-400 g were injected with multiple doses of vaccine (0.2 mL per fish on days 0, 14, 21, and 30 after complete anesthesia via intraperitoneal injection). Blood samples were collected from the fish and the obtained serum was frozen at -70 °C.

Enzyme-linked immunosorbent assay (ELISA) antibody measurement

Forty fish from each group were randomly sampled before vaccination, on December 1 (day zero), and from

January 1 to June, for 6 months after immunization. Blood samples were taken from the tail vein after anesthesia with clove oil, and the samples were kept at room temperature for one hour. Then, they were kept at 4 °C overnight to separate the serum and the samples were stored in sterile tubes at -70 °C until using time. Antibody titer against *Y. ruckeri* was performed using indirect ELISA (Raida et al., 2011; Soltani et al., 2014b). A 96-well plate was used to perform the ELISA. One hundred µL of bicarbonate buffer (0.2 M, pH 9.4) containing crushed *Y. ruckeri* antigen (OD=0.13) was added to each well and the plates were kept overnight at 4 °C. Then, the contents of the wells were discarded to remove any unbound antigen, and the wells were washed three times with washing buffer (400 µL containing 0.05% Tween 20 in phosphate-buffered solution with pH 7.2).

In the next step, 300 µL of blocking buffer (2% bovine albumin serum in phosphate-buffered solution) was added to each well and the plate was incubated for three hours at 4 °C. The wells were stored at 37 °C. After discarding the contents of the wells and washing them again, the serum sample was diluted 1:100 in a secondary dilution buffer (phosphate-buffered saline, 0.05% Tween 20, and 5% bovine serum albumin; pH 7.2) and 100 µL of the diluted serum was added to each well. This step was repeated three times for each sample and the plate was kept at room temperature for one hour. After discarding the contents and washing again, 100 µL of a 1:100 diluted solution of a mouse-obtained anti-salmon polyclonal antibody obtained from Dr. Mohammad Khosravi, Department of Pathobiology, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran, was added to each well, and the plate was incubated for one hour at room temperature. Then, 100 µL of diluted anti-mouse IgG conjugated with peroxidase enzyme was added to each well and then the plates were incubated for one hour at room temperature. The contents were discarded and the wells were washed four times. Then, 100 µL of substrate solution (3,3',5,5'-Tetramethylbenzidine) was added to each well and the plate was incubated for 25–30 min at room temperature in the dark. Finally, 100 µL of stop solution (1N hydrochloric acid) was added to each well to stop the reaction, and the optical density was measured at 450 nm using a spectrophotometer. Immunized rainbow trout serum with *Y. ruckeri* antigen repeatedly and intraperitoneally was used as a positive control, and wells containing only dilution buffer were used as background. The background optical density was subtracted from the optical density of the wells to calculate the actual optical density.

Preparation of blood smear and leukocyte count

Blood smears were prepared simultaneously with blood collection. Blood samples were gently mixed with EDTA (ethylenediaminetetraacetic acid) anticoagulant, and a small amount of blood was placed at the end of a microscopic slide using a capillary tube. The smear was fixed with methanol after drying at room temperature. Giemsa staining was used for cell counting in the laboratory.

Bacterial agglutination titration

For bacterial agglutination, serial dilutions (1:2 to 1:64) of serum samples were prepared, and 500 µL of each dilution was transferred to test tubes. Then, 500 µL of *Y. ruckeri* suspension (9×10^8 cfu/mL) was added to each tube. The tubes were gently shaken and left at room temperature overnight and were evaluated macroscopically.

Statistical analysis

Statistical analysis of the obtained results was performed using SPSS software. One-way ANOVA and Tukey's test were used to determine the significance level of the differences in the data. In all assessments, a significance level of $P < 0.05$ (with 95% confidence).

Results

Water quality parameters

The mean temperature and pH of the pond water were recorded as 17.04 ± 0.24 °C and 7.44 ± 0.02 , respectively, in the experiment (Table 2 and Figure 1). The average dissolved oxygen concentration and the concentrations of ammonia and nitrite were 7.89 ± 0.03 mg/L, 0.01 ± 0.06 mg/L, and 0.02 mg/L, respectively. All water quality parameters were in the safe and acceptable range for the health and growth of rainbow trout.

Differential leukocyte counting

Leukocyte counts were significantly different among the four study groups at 0, 1, 2, 3, 4, and 6 months after immunization ($P < 0.05$). The highest percentage of heterophils was recorded in the control group, followed by the single immersion vaccination (SIV), immersion vaccination plus booster (IVB) dose group, and the intraperitoneal vaccination (IPV) group (Table 3). There was no significant difference between the SIV group and the IVB dose group in February (two months after vaccination) and between the IVB dose group and the intraperitoneal group in March and April (three and four months after

Table 1. Experimental groups and their vaccination method

Groups	Treatments
1	Control (no vaccine)
2	SIV
3	IVB dose
4	Intraperitoneal injection vaccination

vaccination) ($P>0.05$). However, a significant difference was observed between the other groups in April ($P<0.05$). In other cases, the P-Value was calculated to be less than 0.001, indicating an extremely significant difference between the groups at other times during the study.

A significant difference was observed in the percentage of differentiated lymphocytes among the four groups at time points zero, one, two, three, four, five, and six months after the study ($P<0.05$). The highest percentage of lymphocytes was recorded in the intraperitoneal injection vaccination group, followed by the IVB dose group, the SIV group, and the control group, respectively (Table 3). There was no significant difference between the SIV and IVB dose groups in February ($P>0.05$). Similarly, no significant difference was observed in the IVB dose and intraperitoneal injection vaccination groups in March. However, a significant difference was observed between the SIV and IVB dose groups in April ($P<0.05$). In other comparisons, an extremely significant difference was observed ($P<0.001$), which probably reflects the real effects of the variable under study.

The percentage of differentiated monocytes in the control group was significantly higher than in the SIV group in January (30 days after vaccination) ($P<0.05$). There were no significant differences between the other groups during the study period ($P>0.05$, Table 3).

Antibody titer by agglutination method

Antibody titer evaluation showed that the intraperitoneal injection vaccination and IVB dose groups had significantly higher titers than the SIV group at all studied time points ($P<0.05$, Table 4). In addition, the intraperitoneal injection vaccination group showed higher titers than the IVB dose group at all studied time points except for May. The intraperitoneal injection vaccination group showed a significant difference from the SIV group in February ($P<0.05$). The intraperitoneal injection vaccination group showed also significantly higher titers than both the IVB dose in June ($P<0.05$). In addition, the p-value between the intraperitoneal injection vaccination and SIV groups was calculated to be less than 0.001 in June and February. No significant differences were observed in other groups and studied time points ($P>0.05$, Table 4).

ELISA antibody titer

The antibody titer by ELISA in all vaccinated groups was significantly higher than in the control group ($P<0.05$, Table 5). There was no significant difference between the IVB dose group and the intraperitoneal injection vaccination group in February ($P>0.05$), but both groups had significantly higher levels than the SIV group ($P<0.05$). No significant difference was observed between the SIV and IVB dose groups in March

Table 2. Water quality parameters throughout the experiment

Months	Temperature (°C)	Oxygen (mg/L)	Ammonia (mg/L)	Nitrite (mg/L)	pH
December	17	7.8	0.01	0.05	7.4
January	16.8	7.9	0.02	0.05	7.5
February	16.2	8	0.01	0.05	7.5
March	16.7	8	0.02	0.05	7.4
April	16.9	7.9	0.02	0.05	7.4
May	17.6	7.8	0.02	0.05	7.4
June	18.1	7.8	0.02	0.1	7.5

Table 3. Values of immunocompetent cell population in the studied groups post-vaccination

Variables	Months	Control	Immersion	Immersion Plus Booster	Intraperitoneal Injection
Heterophil	December	10.13±0.334	-	-	-
	January	9.88±0.183 ^a	5.1±0.147 ^b	-	-
	February	7.8±0.187 ^a	4.8±0.172 ^b	4.5±0.164 ^b	3.5±0.107 ^c
	March	9.4±0.226 ^a	5.3±0.18 ^b	3.8±0.12 ^c	3.2±0.14 ^c
	April	6.8±0.125 ^a	6.08±0.126 ^b	5.38±0.137 ^c	5.35±0.216 ^c
	May	Not detected	Not detected	Not detected	Not detected
	June	8.25±0.155 ^a	7.5±0.119 ^b	5.78±0.131 ^c	4.15±0.111 ^d
Lymphocytes	December	89±0.367	-	-	-
	January	88.45±0.322 ^a	94.64±0.171 ^b	-	-
	February	91.7±0.227 ^a	94.5±0.164 ^b	95±0.124 ^b	96.1±0.112 ^c
	March	90.05±0.275 ^a	94.55±0.172 ^b	96±0.143 ^c	96.6±0.138 ^c
	April	92.6±0.128 ^a	93.45±0.175 ^b	94.25±0.155 ^c	95.38±0.117 ^c
	May	Not detected	Not detected	Not detected	Not detected
	June	88.35±0.292 ^a	92.23±0.127 ^b	93.78±0.141 ^c	95.48±0.152 ^d
Monocytes	December	0.68±0.11	-	-	-
	January	0.5±0.095 ^a	0.23±0.067 ^b	-	-
	February	0.4±0.078 ^a	0.5±0.107 ^a	0.4±0.078 ^a	0.3±0.073 ^a
	March	0.45±0.087 ^a	0.28±0.071 ^a	0.18±0.061 ^a	0.2±0.064 ^a
	April	0.4±0.078 ^a	0.43±0.101 ^a	0.25±0.069 ^a	0.33±0.075 ^a
	May	Not detected	Not detected	Not detected	Not detected
	June	0.35±0.076 ^a	0.23±0.067 ^a	0.23±0.067 ^a	0.25±0.069 ^a

Note: Different superscript letters (a–d) within each row indicate significant differences among experimental groups ($P < 0.05$), whereas values sharing at least one common letter are not significantly different ($P > 0.05$)

($P > 0.05$), but the immunity level of both groups was significantly higher than the intraperitoneal injection vaccination group ($P < 0.05$). A significant difference between the IVB dose group and the SIV group was observed in April ($P < 0.05$). The IVB dose group showed a higher level of immunity than the other groups in May and June ($P < 0.05$). The antibody titer in the intraperitoneal injection vaccination group was higher than that in the SIV group ($P < 0.05$, Table 5).

Discussion

Yersiniosis is known as a redmouth enterobacterial disease of aquatic animals that affects almost all species

of farmed fish and causes significant economic losses in the aquaculture industry (Tobback et al., 2007; Kumar et al., 2015, Tacon, 2020). Iran has faced a widespread outbreak of this disease since its first report (Soltani et al., 1999). The use of antibiotics has limitations due to the increase in antibiotic resistance and residues in aquatic animals (Ljubojević Pelić et al., 2024). An Iranian study showed that the prevalence of diseases, including yersiniosis, and repeated treatments against these diseases have contributed to increased antibiotic resistance, such as to erythromycin and oxytetracycline (Sultani et al., 2023). Vaccination is considered the most effective strategy to combat antibiotic resistance and control the disease (Austin & Robertson, 2003), but the persistence

Table 4. Antibody titer measured in the serum of rainbow trout in different groups of the study over six months post-immunization using agglutination assay

Months	Immersion	Immersion Plus Booster	Intraperitoneal Injection
January	25.2±2.149	-	-
February	13.4±1.698 ^a	25.8±3.693 ^b	31.45±3.499 ^b
March	14.1±2.268 ^a	14.25±2.306 ^a	23.2±3.549 ^a
April	10.75±1.898 ^a	14.55±2.57 ^a	14.75±2.492 ^a
May	8.95±1.121 ^a	14.7±2.358 ^a	12.8±1.948 ^a
June	7.3±1.099 ^a	14.3±1.805 ^b	22.6±2.635 ^c

Note: Different superscript letters (a-c) in each row indicate statistically significant differences among vaccination groups according to Tukey's post hoc test ($P<0.05$). Means sharing the same letter are not significantly different ($P>0.05$).

of the disease is due to different bacterial biotypes and the limitations of available vaccines. Studies have shown varying efficacy based on factors, such as age, weight, and environmental conditions of the fish. Although short-term safety responses have been well documented, long-term safety has yet to be investigated. Previous research suggests that vaccinations can enhance immune responses against enteric redmouth disease (ERM) (Plant & LaPatra, 2011). Studies show that vaccination methods significantly affect fish immunity with the injection method being one of the most effective (Jaafar et al., 2015; Deshmukh & Raida, 2012).

The findings of the present study provide valuable insight into the immune response induced by the investigated vaccination methods. Serological analyses, such as agglutination and ELISA, demonstrated a strong humoral immune response after vaccination.

Agglutination assays showed a significant increase in IgM immunoglobulin levels in the vaccination groups. The intraperitoneal injection and immersion plus booster dose groups exhibited stronger immune responses than the single immersion group. Moreover, the intraperitoneal and immersion-plus-booster groups showed more sustained immune responses due to the boosting effect, compared with the single immersion group. The IPV group also had higher immunity than the immersion-plus-booster group, likely due to greater antigen exposure and immune activation afforded by the intraperitoneal route, which provides more direct stimulation of the immune system and a prolonged release of antigens.

Agglutination results were higher in the intraperitoneal injection group, while antibody titers by ELISA showed that the IVB dose group also elicited significant humoral responses. This suggests that although the intraperitone-

Table 5. Antibody titer measured by ELISA in the serum of rainbow trout in different groups of the study over six months post-immunization

Months	Control	Immersion	Immersion Plus Booster	Intraperitoneal Injection
December	0.2±0.003	-	-	-
January	0.21±0.003 ^a	0.42±0.017 ^b	-	-
February	0.2±0.004 ^a	0.55±0.014 ^b	0.73±0.039 ^c	0.76±0.024 ^c
March	0.2±0.003 ^a	0.72±0.023 ^b	0.72±0.03 ^b	0.63±0.011 ^c
April	0.2±0.003 ^a	0.74±0.034 ^b	0.87±0.031 ^c	0.79±0.038 ^{bc}
May	0.21±0.004 ^a	0.37±0.01 ^b	0.82±0.031 ^c	0.7±0.022 ^d
June	0.2±0.004 ^a	0.48±0.021 ^b	0.77±0.019 ^c	0.58±0.019 ^d

Note: Means with different superscript letters (a-d) within the same row are significantly different ($P<0.05$). Means sharing one or more common letters are not significantly different according to Tukey's multiple comparison test.

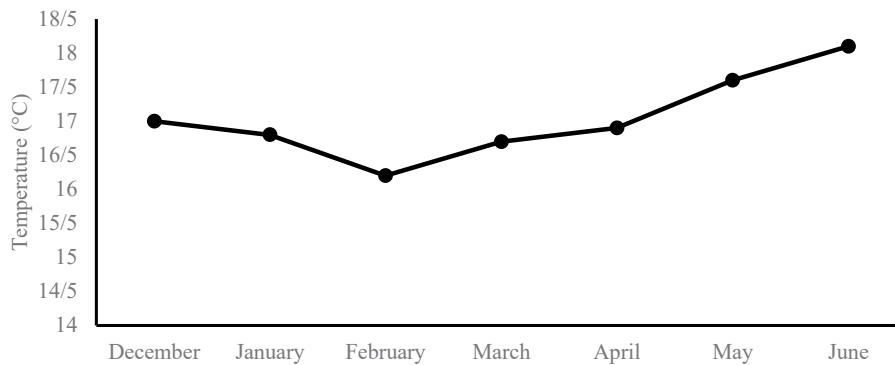


Figure 1. Water temperature fluctuations throughout the experiment

al injection method may initially yield higher antibody levels, the IVB dose method can still effectively stimulate the humoral immune response in fish.

Leukocyte counts provided additional insight into the activation of the immune system in vaccinated fish. High leukocyte levels were observed in both the intraperitoneal injection and immersion vaccination groups, indicating stimulation of the fish's immune response. Leukocyte profiling showed that both vaccination strategies could induce an inflammatory response, which is critical for developing an effective adaptive immune system.

In the present study, different results were observed in the comparison of antibody titers measured by both ELISA and agglutination methods. Both methods indicated that the intraperitoneal injection group had the highest overall antibody titers, followed by the IVB dose group and the SIV group. By contrast, ELISA showed the highest titers in the IVB dose group, while the intraperitoneal and SIV groups had the lowest levels. These differences may be due to the different sensitivities and mechanisms of each method. Although the intraperitoneal injection vaccination method was generally effective in inducing a strong immune response, the IVB dose method provided a significantly long-term immune response, as indicated by the ELISA results. Therefore, based on the findings of the present study, the IVB dose can be recommended for practical aquaculture due to the effectiveness of the immune response and its ease of application.

Consistent with the serological findings, water temperature in the aquaculture environment showed a concomitant increase that peaked around the sixth month after vaccination. Since rainbow trout are a cold-water fish species, the observed increase in water temperature is a common physiological marker of inflammation and immune activation, which further confirms the immu-

nogenicity of the vaccine. However, further studies are required to assess the efficacy of the vaccine by challenging the immunized fish with the virulent strain of *Y. ruckeri*. In this work we could not provide the challenge test due to the lack of an isolated area for performing such a bioassay at the fish farm location.

Overall, the results of the present study showed that the vaccine candidate was able to induce a favorable immune response in rainbow trout. This was confirmed by simultaneous increases in serological markers and leukocyte counts at six months, with corresponding observations of water temperature. A comparison of vaccination methods demonstrated the importance of using both vaccination approaches and booster doses to optimize the immune response of the fish.

The findings of this study contributed to the scientific knowledge on the evaluation and application of veterinary vaccines for cold-water fish species, and the insights gained can be used in the design and evaluation of future vaccine candidates.

Conclusion

The present study provided valuable findings that showed that the vaccine against yersiniosis has a significant impact on the health and survival of rainbow trout. In addition, water quality appears to be an environmental factor that can affect the efficacy of the vaccine. The results of this research can serve as a useful scientific basis for strengthening vaccination strategies and water quality management in aquaculture.

The research findings on the yersiniosis vaccine have important implications for health policies in the aquaculture industry. These findings can help formulate and refine health policies related to vaccination and dis-

ease management. Increasing awareness and education among fish farmers and aquaculture workers is crucial to enhance understanding of the importance of vaccination and disease prevention methods. Furthermore, expanding the use of indigenous *Yersinia* vaccines could reduce dependence on chemical drugs and antibiotics.

Additionally, developing health guidelines and protocols based on these research findings can improve vaccination and disease management practices on farms. Ultimately, the results of the present study can serve as scientific support for adopting national policies on aquatic animal health and disease management, helping to improve public health and reduce disease outbreaks in the aquaculture sector.

Ethical Considerations

Compliance with ethical guidelines

All ethical principles are considered in this article.

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Authors' contributions

All authors equally contributed to preparing this article.

Conflict of interest

The authors declared no conflict of interest.

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