Features of the immune response of rainbow trout to vaccination of antigens Yersinia ruckeri, Aeromonas salmonicida, Listonella anguillarum and Renibacterium salmoninarum

Alexey Droshnev, *Elena* Zavyalova, *Marianna* Karpova, and *Darya* Alontseva*

Federal State Budget Scientific Institution "Federal Scientific Centre VIEV", 24k1 Rjazanskiy prospeckt, Moscow, 109428, Russian Federation

Abstract. The antigens of Yersinia ruckeri, Aeromonas salmonicida sp.salmonicida, Listonella anguillarum and Renibacterium salmoninarum were immunized to rainbow trout of different ages immersion and intraperitaneal, then waited 300 degree days, after which they were infected with homologous and heterologous strains of the studied microorganisms. Prepared antigens and their various combinations were used in the experiments. After the control infection, the level of protective immunity was compared. The level of antibacterial protection obtained by the introduction of polyvalent variants was comparable to that acquired from the introduction of monovalent antigens. A number of cross–reactions were detected - so vaccination with pure A. salmonicida antigen provided some protection against infection with L. anguillarum and Y. ruckeri. In addition, the addition of R. salmoninarum antigen in combination enhanced the protective effect against other diseases to a greater extent than from homologous infection.

1 Introduction

Throughout the history of mankind, fishing has been the most important way to provide people with animal protein, therefore, fish farming is known as one of the oldest areas of economic activity. Back in India, 2000 BC, the foundations of fish cultivation were laid, later, in 599 BC, the first manuals on breeding water bodies were published in China. Russia, until the 80s of the last century, was actively engaged in fishing, and growing in captivity concerned the reproduction of natural reserves of only the most valuable and delicious fish species, that is, it was not commodity-mass. And it was only in the 2000s that the aquaculture of Russia received a certain incentive for development - in particular, in the North–West of the country, rapid development of trout farming began both in fresh and seawater. Intensive cultivation technologies, inevitably associated with high densities and active trade in fish material, have led to the spread of viral and bacterial diseases, as well as fish parasitoses. The active use of antibacterial drugs contributed to the antibiotic resistance of circulating strains

* Corresponding author: livejustonce@yandex.ru

and the transition of diseases to a chronic form. Common sense and foreign experience have shown that only the use of specific prevention will allow you to abandon the use of medications that cause serious side effects.

The Laboratory of Ichthyopathology of the FSC VIEV has been developing biologically active drugs for bacterial etiology of fish diseases since 2006. Vaccines have been developed against classical vibriosis (*Listonella (Vibrio) anguillarum*), which is inevitable when growing fish in mariculture; yersiniosis (*Yersinia ruckeri*), which some time ago became a serious problem in the Karelia aquaculture [1]. Available drug routes of administration (immersion, injection, oral (for revaccination)), as well as the administration of other nosological units (*Aeromonas salmonicida, Flexibacter columnaris., Flexibacter psyhrophila, Moritella viscosa, Vibrio salmonicida*) were successfully tested.

However, in modern conditions, the use of a monovalent vaccine planned for the transportation of fish to a threatened zone is not always justified. The mobility of aquaculture facilities leads to the need to combine several bacterins in one preparation. This will make it possible to plan economic activities more efficiently using the potential of both fresh and sea water for cultivation. In addition, the world practice of vaccination prevention has come to use polyvalent variants of drugs [2], since the introduction of injectable vaccines to fish is a high-tech process, and it is more profitable to organize protection against several diseases in one action. Combinations of different forms of vaccines are also interesting from a scientific and practical point of view. For example, immersion and injection, injection and oral revaccination, etc., in order to further increase their effectiveness.

However, the use of combined vaccines may be limited by mechanisms of internal interference or inhibition of the antibody formation system in fish with simultaneous administration of antigens derived from different bacterial species or serotypes.

The aim of this study is to evaluate the effect of combinations of antigens *Yersinia ruckeri, Aeromonas salmonicida, Listonella anguillarum* and *Renibacterium salmoninarum* on the immunity of salmon in comparison with monovalent bacteria to create a truly effective drug.

2 Materials and methods

Bacterial strains. The bacterins *Listonella (Vibrio) anguillarum* of serotype 01 (VIEV AF ¾) and serotype 02 (VIEV VBF 07), *Y. ruckeri* serotype I (strain "RK 7No.3-10"), *A.salmonicida* (strain "RK-Gong-10"), *R.salmoninarum* (strain "KIM-16") were used from the collection of the Laboratory of Ichthyopathology of the FSC VIEV.

Microorganisms were grown in fish-peptone broth and Hottinger broth without and with a content of 10 % seawater, with an amine nitrogen concentration of at least 180 mg%. Cultivation was carried out parallel to each other in the fermenter to the maximum level of accumulation of microbial cells in the growth medium (from $4x10^9$ m.cells / cm3). *R.salmoninarum* was accumulated both under controlled reactor conditions and on an agar multilayer substrate using liquid and dense media of its own design, including growth factors, L-cysteine and rainbow trout serum. In the future, the microbial mass was inactivated, followed by control and washing of microbial cells from ballast components.

Individual containers with the bacterial mass of each strain were inactivated with formalin to 0.37% and subjected to 3-fold re-precipitation by centrifugation of antigens in 0.15 M phosphate-salt buffer pH 7.6 (standard composition). The completeness of inactivation was determined by the absence of microbial growth on nutrient media such as tryptose-soy agar and broth, thioglycol medium, Saburo agar, TCBS [3, 4], KDM-2, SKDM [5]. The optical concentration of cells was determined by the spectrophotometric method using a calibration curve.

The obtained antigens of each pathogen with a concentration of 0.5×10^7 – 0.75×10^8 m. c./cm^3 are taken in a mixture in equal volume, aluminum hydroxide (6% gel) in a volume of 12% as an adjuvant, followed by dispersion on a rotary unit for 30 minutes.

The concentration of the antigen in terms of microbial cells for immersion vaccination was 2×10^8 m.c./cm^3 with the possibility of processing up to 10 kg of live fish in a working solution.

Fish. For research in the conditions of the aquarium laboratory, 3180 specimens of rainbow trout (*Oncorhynchus mykiss (Walbaum*) with a weight from 6 to 35 g were used. All the fish were delivered in the form of fertilized eggs from a fish farm that was safe for infectious and invasive diseases, incubated and grown in an aquarium environment, in an environment free of pathogens. The temperature of the content was 14-16°C, and the fish were kept at least 300 degrees days after vaccination before they were subjected to a control infection.

The experiments were carried out at a temperature of 16°C, bacterins and their combinations were injected or by immersion of fish in a working solution of the vaccine for 120 seconds. (series 1) or intraperitoneally in a volume of 0.1 cm^3 per head (series 2,3,4).

Survival after infection was used to assess the effect of immunization, and the cause of death was confirmed by re-isolation from fallen individuals on nutrient media of a specific agent used in a specific experiment. The number of fish in the group was at least 30.

Bioassay. Fish were infected by intraperitoneal injection with bacterial culture at a dose of 0.1-0.2 cm^3 with a concentration of LD_{100} for each strain. The experimental fish were monitored for 14 days. Isolation of the initial strains from infected fish was carried out according to the generally accepted method.

To study the protective properties when using polyvalent variants, a series of experiments were conducted in which the survival of fish after a control infection was evaluated, conducted 300 degree days after the administration of the studied compositions. To control the effectiveness of protection, vaccinated monovalent variants and intact (unvaccinated) fish were infected according to the same scheme.

Different combinations of bacterins were used in four experiments.

Series 1: combination of *L. anguillarum* (two strains) and *Y.ruckeri*;

Series 2: Combination of *Y.ruckeri* and *A.salmonicida*;

Series 3: Combination of *Y.ruckeri, A.salmonicida* and *R.salmoninarum*;

Series 4: combination of *L.anguillarum* (two strains), *Y.ruckeri*, *A.salmonicida* and *R.salmoninarum*.

Statistical studies were carried out using the Pearson's chi-squared test [6].

3 Results and discussion

The results of the series studies are presented in Tables 1,2,3,4.

It follows from Table 1 that mortality in the control groups of infected rainbow trout, which, according to the results of the experiment and the isolation of the pathogen on nutrient media, were diagnosed with vibriosis and yersiniosis, was 100%. Maximum protection was provided in groups vaccinated with *L. anguillarum* bacterin (2 strains) when infected with homologous strains. There was no protection against heterologous infection from *Y. ruckeri*. Similarly, *Y. ruckeri* bacterin did not provide protection against heterologous strains of *L. anguillarum*, while the minimum number of individuals killed in the bioassay was observed when infected with yersinia. The combined drug provided significant protection against all infections, and the level of protection was comparable to the level obtained by using a monovalent bacterin of each group.

Table 1. Effect of a combination of Listonella anguillarum and Yersinia ruckeri antigens on the development of protective immunity in rainbow trout weighing 6 g. Immersion vaccination (120 sec.) and control infection 300 days after vaccination.

Control challenge		Antigen			Control
		Listonella anguillarum	***Yersinia ruckeri***	***Combination***	
Listonella anguillarum serotype 1	Total (spec.)	30	30	40	30
	Dead (spec.)	8	27	12	30
	Protection/mortality (%)	73.3/26.7	10/90	70/30	0/100
Listonella anguillarum serotype 2	Total (spec.)	30	30	40	30
	Dead (spec.)	9	28	11	30
	Protection/mortality (%)	70/30	6.6/93.4	72.5/27.5	0/100
Yersinia ruckeri	Total (spec.)	40	40	50	40
	Dead (spec.)	38	10	15	40
	Protection/mortality (%)	5/95	75/25	70/20	0/100

P≤0.01 (correlation between clinical signs is statistically significant)

Table 2. Effect of the combination of Yersinia ruckeri and Aeromonas salmonicida antigens on the development of protective immunity in rainbow trout weighing 25 g. Injection vaccination and control infection 300 degree days after vaccination.

Control challenge		Antigen			Control
		Yersinia ruckeri	**Aeromonas salmonicida**	**Combination**	
Yersinia ruckeri	Total (spec.)	50	50	75	50
	Dead (spec.)	8	42	12	50
	Protection/mortality (%)	84	16	84	0
Aeromonas salmonicida	Total (spec.)	50	50	75	50
	Dead (spec.)	40	10	15	50
	Protection/mortality (%)	20	80	80	0

P≤0.01 (correlation between clinical signs is statistically significant)

The data in Table 2 show that maximum protection was provided in groups of fish vaccinated with bacterin and infected with a strain homologous to it. The combination of Y. ruckeri and A. salmonicida bacterins did not reduce protection against homologous infection, providing 84 and 80%, respectively. Mortality in the unvaccinated control groups was 100%.

The data in Table 3 confirm that Y. ruckeri and A. salmonicida antigens provided maximum protection in groups vaccinated with both monovalent and polyvalent variants. The percentage of protection was at the level of 82-95%. R.salmoninarum is characterized by partial protection in both mono- (48%) and polyvalent composition (45%). Especially important is the information that the use of a polyvalent vaccine of three main components (Y. ruckeri, A. salmonicida, R.salmoninarum) provides enhanced immune protection against infection with Y. ruckeri, A. salmonicida strains and amounts to 92-95%.

In the experiment, the results of which are presented in Table 4, it was found that the immunization of fish with a pentavalent vaccine provided 91 and 90% protection against infection with vibrions and yersinia, which is more than the administration of these monobacterins (84 and 86%, respectively). While the combination provided better protection against furunculosis (95%) than vaccination with the monovalent bacterin A.salmonicida (80%).

Table 3. Effect of the combination of Yersinia ruckeri, A.salmonicida and R.salmoninarum antigens on the development of protective immunity in rainbow trout weighing 35 g. Injection vaccination and control infection 300 degree days after administration.

Control challenge		Antigen				Control
		Y. ruckeri	*A. salmonicida*	*R. salmoninarum*	*Combination*	
Y. ruckeri	Total (spec.)	50	50	50	100	50
	Dead (spec.)	8	44	48	5	50
	Protection (%)	84	12	4	95	0
A. salmonicida	Total (spec.)	50	50	50	100	50
	Dead (spec.)	42	9	48	8	49
	Protection (%)	16	82	4	92	2
R. salmoninarum	Total (spec.)	50	50	50	100	50
	Dead (spec.)	30	32	26	55	37
	Protection (%)	40	36	48	45	26

P≤0.01 (correlation between clinical signs is statistically significant)

Table 4. Effect of a combination of antigens Listonella anguillarum (two strains), Yersinia ruckeri, A.salmonicida and R.salmoninarum on the development of protective immunity in rainbow trout weighing 35 g. Injection vaccination and control infection 300 degree days after administration.

Control challenge		Antigen					Control
		L. anguillarum serotype 1, 2	*Y.ruckeri*	*A.salmonicida*	*R.salmoninarum*	Combination	
L. anguillarum serotype 1, 2	Total (spec.)	50	50	50	50	100	50
	Dead (spec.)	8	48	48	47	9	50
	Protection (%)	84	4	4	6	91	0
Y. ruckeri	Total (spec.)	50	50	50	50	100	50
	Dead (spec.)	46	7	48	46	10	50
	Protection (%)	8	86	4	8	90	0
A. salmonicida	Total (spec.)	50	50	50	50	100	50
	Dead (spec.)	44	45	10	45	5	50
	Protection (%)	12	10	80	10	95	0
R. salmoninarum	Total (spec.)	50	50	50	50	100	50
	Dead (spec.)	48	47	44	26	56	49

	Protection (%)	4	6	12	48	44	2

P≤0,01 (correlation between clinical signs is statistically significant)

At the same time, the administration of the *R.salmoninarum* monoantigen and subsequent infection did not reveal a significant level of protection against furunculosis, vibriosis and yersiniosis. Protection against homologous infection was 48% and with polyvalent use 44%.

However, the enhancing vaccination effect of adding R.salmoninarum antigen to the composition was expressed in all experimental groups.

4 Conclusion

The presented results of studies performed on a large number of experimental fish showed that polyvalent compositions provided significant protection against the studied infections, and the degree of protection was comparable or exceeded the level obtained by using a monovalent antigen. It is obvious that with the introduction of monovalent bacterins, protection against a specific pathogen is provided to one degree or another, but the immune system of fish is such that the addition of other antigens to the drug, for example, the *R. salmoninarum* component, provided increased immune protection against infection with *L. anguillarum, Y. ruckeri, A. salmonicida* strains. In this study, we provide only information about the results of direct infection – as the most indicative, without using for analysis knowledge about the titers of serum antibodies that were determined in fish in each group before the biological test (information will be published separately), so as not to mislead when determining protective immunity. Since there is a lot of information in the literature that fish vaccinated with bacteria *V. anguillarum* or *Y.ruckeri*, there are no high titers of serum antibodies, but they are significantly protected for more than 1 year [7, 8]. Conversely, fish vaccinated with *A. salmonicida* bacteria by injection may exhibit high antibody titers, but are not protected from virulent infection [9, 10].

Therefore, the effectiveness of any vaccines must be confirmed only by contact with the pathogen. The polyvalent variants in our study showed no signs of decreased protection, but several cases of heterologous cross-reaction were identified. For example, *A. salmonicida* bacteria provided incomplete but additional protection against vibriosis, and the combination of *A. salmonicida* and *R.salmoninarum* showed values of protection against furunculosis exceeding the homologous monovalent bacterin *A. salmonicida*. It has also been found that the addition of *R.salmoninarum* antigen to other combinations enhances the protective effect of other components. This mechanism requires additional study.

Currently, many medical and veterinary biologics are polyvalent and are effectively used. The information we have obtained shows that multicomponent vaccines for aquaculture will be in demand in the field of fish health protection. This will make it possible to abandon many chemotherapeutic agents and carry out prolonged prevention of mass diseases.

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