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IMMUNIZATION OF RAINBOW TROUT (ONCORHYNCHUS MYKISS-WALBAUM) WITH AEROMONAS SALMONICIDA BACTERIN

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Rainbow trout yearlings (Oncorhynchus mykiss-Walbaum) with the average weight of about 100 g were vaccinated by hyperosmotic infiltration or intraperitoneally with Aeromonas salmonicida in saline and in oil adjuvant. The value of hemagglutinating antibodies was followed in the period of 30-45 days after vaccination and revaccination in treated and control unvaccinated fish.

Challenge infection was carried out in all experimental groups. Each fish was injected with pathogenic Aeromonas salmonicida microorganisms (0,1 ml i/p of $3x10^8$ C. F. U./ml).

Two weeks after the challenge 73,11% of the control fish died. It was observed that mortality was lower in both vaccinated groups. It seems that protection was related to the presence of antibodies and the method of vaccination. Intraperitoneal vaccination was most favourable.

Key words: furunculosis, vaccine, hyperosmotic infiltration, intraperitoneal vaccination, rainbow trout.

INTRODUCTION

Furunculosis in rainbow trout is an endemic disease in Yugoslavia and many studies have been made on it. In particular, research concentrates on the possibility of making a vaccine against this infectious disease and on the method of vaccination. Oral vaccination (Corbel 1975) and intraperitoneal (IP) vaccination with or without adjuvant (Krantz et al. 1963, 1964; Overholser 1968; Paterson and Fryer 1974, Jeremić 1989; Jeremić et al. 1997), have been used. Laboratory and field trials had some success, but because of the variability of results and difficultes in application, the vaccines were not commercialised.

According to some papers IP application is an acceptable method. New methods include hyperosmotic infiltration (HI), simple immersion in a bath with a solution of vaccine and spray vaccination. These technics have been completely developed for vaccination against vibriosis and yersiniosis.

Our research considered a field attempt of vaccination against furunculosis by hyperosmotic infiltration and IP inoculation with the adjuvant. Using the serum of immunized and control groups of fish, we followed immunological reactions, by a classical agglutination method.

The results of vaccination were verified by exposing specimens of rainbow trout to artificial infection with pathogenic Aeromonas salmonicida bacteria.

MATERIAL AND METHODS

Experimental fish

Rainbow trout yearlings (Oncorhynchus mykiss-Walbaum) with the average weight of about 100 g, from common breeding stock, were held in fish ponds.

The preparation of vaccine

Vaccine was prepared from Aeromonas salmonicida isolated during numerous attacks of furunculosis in the Republic of Serbia. The strains were aggregate forms. Vaccine and antigen for the agglutination test were prepared by cultivation of the organisms in tryptose soya bullion (TSB Torlak) mixed by a magnetic mixer for better aeration at 20^{0}C for 48h. The microorganisms were simultaneously seeded on tryptose soya agar (TSA) to check their purity. Prior to inactivation of the bacterial culture in formalin, we took 1 ml of mature bullion culture to determine the number of colonies (C. F. U.). The number of colonies was calculated by spreading on plates (in triplet) 1 ml of serial10-fold dilutions of the cultures in physiological saline on TSA. After 48 h, each culture was inactivated by gradual addition of formalin (final concentration 0,4%) with further incubation at 20^{0}C for 24h. Sterility was tested by inoculation of TSB, TSA, and blood plates with a sample of the inactivated culture. Plates seeded in this way were incubated at 20^{0}C. The plates were examined 24, 48,and 72h after seeding.

In this way, vaccine for hyperosmotic infiltration was prepared

For intraperitoneal (IP) vaccination, inactivated whole cells of A. Salmonicida were collected from the bullion by centrifugation at 3000 rpm for 30 min., rinsed twice with sterile 0,85% saline and resuspended in 0,85% saline to Mac Farland 2 cell concentration. The vaccine was prepared with Freund's complete adjuvant (VCA: GIBCO) as an emulsion. A simple emulsion of iFCA cell suspension (1:1) was emulsed to the second phase with an equal volume of sterile 2% Tween 80 (BDH) in 0,85% physiological saline. The emulsion was microscopically tested to confirm uniformity. There was no free liquid in this emulsion. Vaccine prepared in this way was seeded on TSA and blood plates in order to check for sterility. Sterilised vaccine was stored at 4^{0}C until use.

Antibody titer

Fish blood samples were taken with a syringe from the caudal artery. The blood was left to coagulate at room temperature. Serum was separated by centrifugation and kept at - 20^{0}C. Samples were taken from 20 fish of each experimental group prior to vaccination, to establish the starting antibody titer, then 30 days after vaccination, and 15 days after revaccination. The titer of

agglutination antibodies was determined by the classical agglutination method using 0,5 ml of twofold serial dilutions of serum in sterile physiological saline added to an equal volume of inactivated cell suspension of Aeromonas salmonicida. The mixture of serum and antigen was incubated overnight at +4^{0}C, then for 4h at 20^{0}C. In reading the results, attention was paid to the nature and intensity of agglutination.

Vaccination procedure

Hyperosmotic vaccination

For hyperosmotic infiltration, 100 rainbow trout yearlings were taken. The first experimental group was preliminarily immersed in 8% sodium chloride (NaCl) solution for 2 min., and then 5 min., in a bath with vaccine from formalinised A.salmonicida. For this group, 4 l of vaccine was prepared, and diluted with 200 l of water. During the hyperosmotic vaccination, fish were continuously aerated, and after that returned to the pool.

Intraperitoneal vaccination

For intraperitoneal vaccination, 100 rainbow trout yearlings were taken.

Fish of this experimental group were preliminarily anesthetized with MS 222 (Sandos). Each fish was taken separately by hand and vaccinated intraperitoneally with 0,1 ml of adjuvant - containing vaccine using a 1 ml Bacton and Dickinson automatic syringe with a 25 G needle.

The third experimental group of 100 rainbow trout yearlings was left untreated as a control.

Revaccination

Revaccination was conducted 30 days after vaccination in exactly the same was as vaccination.

Pathogenicity testing procedure

Challenge infection was carried out in the experimental groups by means of injection 15 days after revaccination. Each fish was intraperitoneally injected with pathogenic Aeromonas salmonicida microorganisms (0,1 ml of 320x10^8 C.F.U./ml per fish). The behavior of the fish following artificial infection was monitored for a period of 15 days after the infection. During this time, fish were removed before death and tested for the presence of Aeromonas salmonicida on the basis of colony morphology and agglutination by rabbit anti Aeromonas salmonicida serum. Total mortality of the fish was determined at the end of the two-week observation period.

RESULTS

Hyperosmotic vaccination in two different baths took 20 min.

Intraperitoneal vaccination by two inoculators, took one hour in all. The behavior of all fish returned to normal within 5 min. after their return to the pools.

The results of classical agglutination tests 30 days after vaccination and 15 days after revaccination, are shown in Tables 1 and 2 and Figure 1. The majority

of rainbow trout specimens possessed measurable titers of agglutinating antibodies to furunculosis prior to vaccination. The geometric mean titer (GMT) of antibodies in the control fish was 1:2,45. In the vaccinated groups, a significant increase in the titer of agglutinating antibodies was achieved within four weeks.

The geometric mean titer (GMT) in the hyperosmotic vaccinate group ranged upwards from 1:4,95, which was a 5,7 fold increase of antibody titer in relation to the control. (Table 1). The geometric mean titer (GMT) in the IP vaccinated group ranged upwards from 1:5,45, which was an eight fold increase of antibody titer in relation to the control.

Table 1 The classical agglutination test of HI and IP vaccinated control fish

Treatment method	Number of trout in group	Number of tested trout	Days after injection	Distribution of titres Log^2								GSU Log^2	Anti Log	Increased titre compared with control group
				0	2	3	4	5	6	7	8			
HI*	100	20	30				1	3	12	4		4.95	30.0	5.7
IP**	100	20	30				4	7	6	2	1	5.45	43.0	8
K***	100	20	30	4	6	5	3					2.45	5.5	

HI* - hyperosmotic infiltration
IP** - intraperitoneal vaccination
K*** - control

Two weeks after revaccination the geometrical mean titer (GMT) of antibodies in the hyperosmotic vaccinated group was 1:5,90. Thus the titre was increased 11 times, compared with the control group, and two times compared with the vaccinated group two weeks after revaccination, the geometrical mean titer (GMT) of antibodies in the intraperitoneally vaccinated group was 1:6,80. This the titre was increased 19,9 times compared with the control group, and 2,5 times compared with the vaccinated group.

In the IP vaccinated group, the titer of antibodies was 0,5 units higher than in the HI vaccinated group.

Table 2 The classical agglutination test of revaccinated and control fish

Treatment method	Number of trout in group	Number of tested trout	Days after injection	Distribution of titres Log^2								GSU Log2	Anti Log	Increased titre compared with control group	Increased titre after revaccination
				0	2	3	4	5	6	7	8				
HI*	100	20	15				3	4	7	4	2	5.90	59.7	11	2
IP**	100	20	15					1	8	5	9	6.80	115.4	19.9	2.5
K***	100	20	15	4	6	5	3	2				2,4.5	5.5	5.5	

HI* - hyperosmotic infiltration
IP** - intraperitoneal vaccination
K*** - control

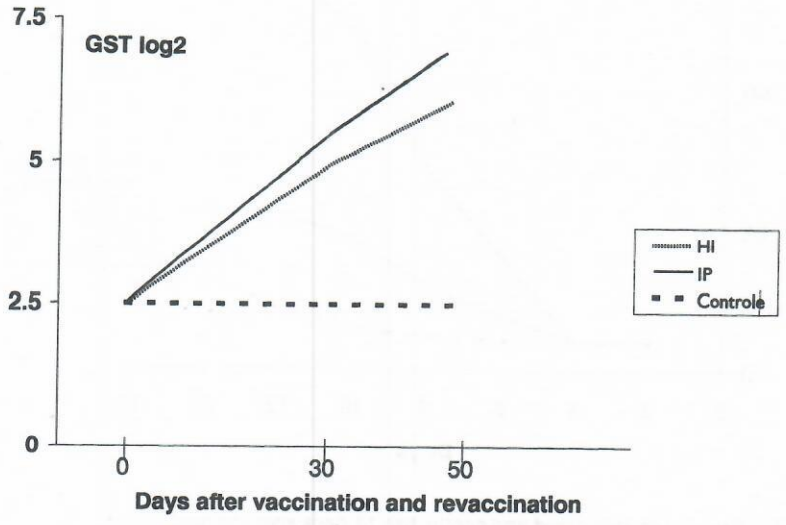

Figure 1. Classic agglutination test in fish after vaccination and revaccination

The percentage mortality of the vaccinated and control fish 14 days after challenge is shown in Table 3 and Figures 2 and 3.

Table 3 Mortality of vaccinated and control fish 14 days after challenge

Treatment	Number of fish	Total mortality and % mortality		Mortality and % mortality from furunculosis	
		number	%	number	%
HI*	87	29	33.33	24	27.58
IP**	92	24	26.08	14	15.21
K***	93	68	73.11	68	73.11

HI* - hyperosmotic infiltration
IP** - intraperitoneal vaccination
K*** - control

Intraperitoneally vaccinated fish had a lower mortality than hyperosmotic vaccinated fish.

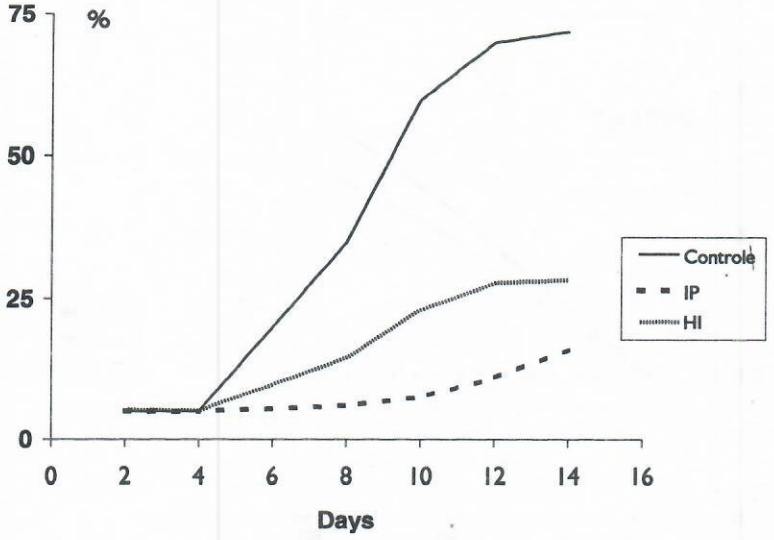

Figure 2. Mortality of vaccinated and control fish 14 days after challenge

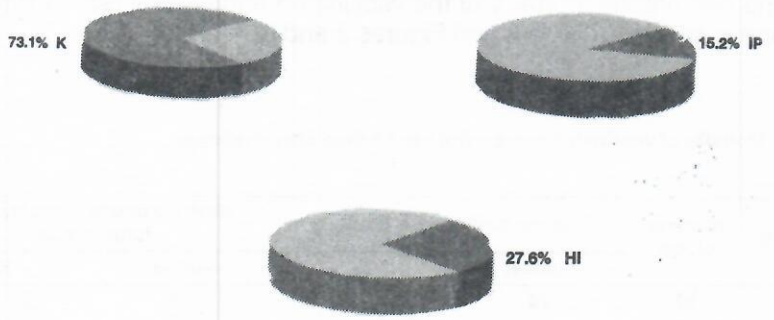

Figure 3. Mortality of vaccinated fish, and fish in the control group 14 days after challenge

Comparing the relative level of protection (RLP), we found that intraperitoneal vaccination gave a greater level of protection (79,20), compared with hyperosmotic vaccination (62,28). The relative level of protection (RLP) between unvaccinated control and different vaccinated groups is shown in Table 4.

Table 4. Comparison of relative level of protection (RLP) between the unvaccinated control and different vaccinated groups.

Treatment method	Number vaccinated	Challenge day	Mortality (%)		RLP
			Vaccinated	Control	
HI	100	45	27.58*	73.11	62.28
IP	100	45	15.21	73.11	79.20

$$RLP = 100 - \frac{\text{\% of mortality of vaccinated}}{\text{\% of mortality of control}} (100)$$

DISCUSSION

Furunculosis is a disease which increases losses in trout farming. The most efficient and the most effective way to reduce such losses is by immunoprophylactic measures with the provision of satisfactory zoohygienic conditions and correct technology.

A significant degree of protection against furunculosis may be achieved with intraperitoneal administration of adjuvanted vaccine and with hyperosmotic vaccination in rainbow trout. According to Fijan (1984), McCarthy (1983), Evelin (1984), and Adams et al. (1988), the strongest immunological response is obtained by intraperitoneal application. The highest level of antibodies in our experiments were achieved by vaccination with multiple doses (revaccination). The obtained results support the conclusions of Johnson and Amend (1984), who found that a higher efficiency of vaccination is achieved by multiple bathing of fish in vaccine.

Hyperosmotic vaccination seemed very promising before it was possible to assume which method of mass-immunization will be the most efficient, and which will provoke the smallest stress in fish (Amend and Fender 1976, Antipa and Amend 1977, Antipa et al. 1980). Later results of Antipa et al. (1980) also indicated that bathing of fish in hypertonic solution had no significant influence on the results of vaccination, and that it provoked strong stress as well. Because of that, this method is not better than others.

However, our results clearly indicate that two hyperosmotic vaccinations with inactivated vaccine, at an interval of 6 weeks, give a satisfactory antibody titer. This finding agrees with the results of Smith et al. (1980).

They also found that the level of circulating antibodies in blood was significantly higher in the hyperosmotically vaccinated group than in the control group.

In our experiments protection from artifical infection with the pathogenic species A. salmonicida, hyperosmotic vaccination was considerable, because the RLP of vaccinated fish was 66,66%. These results contradict those of Palmer and Smith (1980) who found a low level of agglutinating antibodies against furunculosis in hyperosmoticly vaccinated Atlantic salmonids in ponds. That level was not conspicuosly different from the one in the control group.

However, as in our experiments, the results of the above mentioned authors showed an important difference of mortality between the HI vaccinated and control groups during natural infection. They found a significant level of protection against furunculosis using HI vaccination, but no link was found between the titer of agglutinating antibodies for furunculosis and protection against furunculosis.

In comparison with IP vaccination hyperosmotic vaccination provided a lower titer of antibodies ad a lower level of protection.

Our results likewise clearly indicate that high titers of agglutinating antibodies are achieved in vaccinated fish after intraperitoneal vaccination and revaccination at an interval of four weeks. These findings agree with the results of Spence et al. (1965) and Hara et al. (1976). The vaccinated groups of fish in their and our experiments had relatively high agglutinin titers which ranged from 1:320 to 1:640 whereas low agglutinin titers (1:20 to 1:40) were found in the control groups of fish.

The results of Palmer and Smith (1980) also indicate that intraperitoneally vaccinated salmon show a significantly greater increase in antibody titer in comparison with the control groups 11 weeks after vaccination.

However, no difference of mortality between the control and intraperitoneally vaccinated groups was found during natural infection of the fish even through intraperitoneally vaccinated fish showed a significantly greater increase of antibody titer in comparison with the control groups. Thesc authors found to link between a high titer of (agglutinin) antibodies to Aeromonas salmonicida and protection against natural infection. Corbel (1975) cites data indicating that the serum agglutinin titer is not necessarily a reliable index of immunity.

Our results contradict those of the above mentioned authors, since intraperitoneal vaccination ensured a higher percentage of survival after artificial infection than in the control. Thus, intraperitoneal vaccination proved to be very effective against artificial infection with the pathogenic species Aeromonas salmonicida since it protected 73,91% of the vaccinated fish. The control unvaccinated fish had a high percentage mortality (73,11%).

From this, we can conclude that intraperitoneal vaccination provided a significantly higher antibody titer and a significantly lower percentage of death in comparison with the hyperosmotic vaccination and control.

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IMUNIZACIJA KALIFORNIJSKE PASTRMKE (ONCORHYNCHUS MYKISS WALBAUM) AEROMONAS SALMONICIDA BAKTERINOM

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SADRŽAJ

U svrhu imunoprofilakse kalifornijskih pastrmki protiv furunkuloze pripremljena je vakcina na osnovu inaktivisanih antigena A. salmonicida. Sojevi A. salmonicida izolovani su iz parenhimatoznih organa (bubreg, jetra, slezina) kalifornijske pastrmke obolele od furunkuloze.

Ispitivan je uticaj hiperosmotske infiltracije i IP vakcinacije na serološki odgovor vakcinisane kalifornijske pastrmke, te protektivni efekat primenjenih vakcina nakon veštački izazvane infekcije.

Zaključeno je da smo pri različitim metodama vakcinacije (HI i IP) dobijali uvek viši titar antitela i znatno manji procenat uginuća od onog u kontrolnim grupama, mada visina imunog odgovora i procenat preživljavanja je bio najbolji pri intraperitonealnoj vakcinaciji. Takođe smo utvrdili da HI i IP vakcinacija doprinosi dobrom serološkom odgovoru i da štiti vakcinisanu ribu od izlaganja patogenom terenskom izolatu bakterije Aeromonas salmonicida.