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PREPARATION OF A VACCINE FOR INTRAPERITONEAL APPLICATION AGAINST FURUNCULOSIS OF RAINBOW TROUT

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For immunoprophylaxis against furunculosis of rainbow trout, a vaccine has been prepared on the basis of inactivated antigens of A. salmonicida. Strains of A. salmonicida were isolated from parenchymal organs (kidney, liver, spleen) of rainbow trout suffering from furunculosis and were cultured on tryptose soya agar (TSA).

The influence of intraperitoneal vaccination on the serological response of vaccinated rainbow trout was tested as well as the protective effect of the employed vaccine after natural and artificially induced infection.

It may be concluded that intraperitoneal vaccination of fish promotes a good serological response and protects the vaccinated fish from exposure to a pathogenic field isolate of the bacteria A. salmonicida.

Key words (vaccine, furunculosis, californian trout)

INTRODUCTION

Furunculosis is a contagious disease of a septicaemic nature with a peracute, acute, subacute, or chronic course. It predominantly attacks salmonid fish. The disease is caused by the bacterial species, Aeromonas salmonicida. As with other farmed species, the dense population necessary for economic profit on the farms creates an ideal environment for rapid transmission and spreading of disease. The losses caused by seasonal attacks of disease can be enormous, and this makes it imperative to introduce control measures.

Vaccination, or the intentional introduction of components of a disease agent into the organism of an animal, promotes the development of immunity, as a result of which the organism becomes resistant to renewed contact with the same agent (Busch,1978; Fijan, 1984). As one of the methods in combating the spread of infectious diseases, vaccination has played an important role in human and veterinary medicine (Amend, 1976; Rohovec et al., 1975). The development and use of vaccines in veterinary medicine is of great significance because it facilitates the raising of animals in intensive production.

Immunization of fish should become a customary method of preventing fish diseases and would render production more economical by reducing losses (Amend, 1981; Busch, 1978).The case for vaccination is also supported by occasional failures or uncertain outcomes following treatment of diseased fish with hemotherapeutics or antibiotics.

Immunoprophylaxis should be conducted together with general measures involving hygiene, housing, improvment of genetic composition of the population, adherence to health regulations, and proper nutrition (Amend, 1976; De Kinkelin, 1984).

There are differences in the vaccination of mammals and fish, due to the pronounced variability of the immunological response in fish. This applies in particular to the local response, which is more weakly developed and harder to measure.

Differences exist in the ways of administering vaccines to fish,which are as follow: the parenteral way, through injections with or without Freund's adjuvant; oral administration; immersion or bathing in a solution of vaccine; hyperosmotic infiltration; and the spray method.

The greatest immunological response and longest-lasting resistance to the agent are achieved by intraperitoneal (i/p) application of antigen. Such vaccination is superior to other technicques such as immersion or the spray method (Fijan, 1973; Evelyn, 1984). It is generally known that fish are capable of acquiring immunity as demonstrated by serological tests based on the agglutination method in which the presence of agglutinating antibodies is established. It is most often used in diagnosing fish diseases.

Intraperitoneal vaccination of rainbow trout with inactivated Aeromonas salmonicida bacteria was performed in the present work. Immunological reactions were checked by the method of classical agglutination with the serum of immunized and control groups of fish.

The results of vaccination were verified by exposing specimens of rainbow trout to pathogenic Aeromonas salmonicida bacteria and to natural attacks of the disease.

MATERIAL AND METHODS

Vaccine was prepared from A. salmonicida isolated during numerous attacks of furunculosis in the Federal 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 on a magnetic mixer for better aeration at 20^0C for 48 h. The microorganisms were simultaneously seeded on 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 serial 10-fold dilutions of cultures in physiological saline on tryptose soya agar (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 24 h. 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 72 h after seeding.

For intraperitoneal (i/p) 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 (VC: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. Sterile vaccine was stored at +4^{0}C until use.

Blood sampling for the agglutination test. 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, 15 days after revaccination and 4 months after vaccination. 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. Four experimental groups were involved in the experiment:

Experimental group I-100 rainbow trout yearlings;

Experimental group II-100 mature specimens of rainbow trout;

Experimental group III-100 rainbow trout yearlings;

Experimental group IV - 200 mature specimens of rainbow trout.

Fish of the first two experimental groups were preliminarily anesthetized with MS 222 (Sandos).

Each first two experimental groups were preliminarily anesthetized with MS 222 (Sandos). Each fish was taken separately in hand and vaccinated intraperitoneeally with 0,1 ml of adjuvant-containing vaccine using a I ml Becton and Dickinson automatic syringe with a 25 G needle.

The third experimental group was our control. These fish were intraperitoneally injected with 0,1 sterile physiological saline.

The fourth experimental group was also a control. Fish of this group were bathed in pool water without vaccine administration.

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

Pathogenicity testing procedure biological experiment - challenge infection. Challenge infection was carried out in experimental groups I and III by means of injection. Each fish was intraperitoneally injected with pathogenic Aeromonas

salmonicida microorganisms (0,1 ml of 3x10 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.

The fish in experimental groups II and IV, i. e. mature specimens of rainbow trout, were left to be exposed to natural infection after spawning, when furunculosis usually appears in these categories in the fish pond.

RESULTS

Intraperitoneal vaccination by two inoculators took two hours in all. The behavior of all fish returned to normal with in 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.

Table 1. The classical agglutination test of i/p vaccinated and control fish 30 days

Treatment method	Number of trout in group	Number of tested trouts	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			
I/P*	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	

I/P* - intraperitoneal vaccination
K** - control

Two weeks after revaccination, the geometrical average titre of antibody was 1:6.80. Thus the titre was increased 19.9 times, compared with the control group, and 2.5 times compared with the vaccinatee group, two weeks earlier.

Table 2. The classical agglutination of vaccinated and non-vaccinated fish 15 daus after revaccination

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	Increased titre after revaccination
				0	2	3	4	5	6	7	8				
I/P*	100	20	15					1	8	5	9	6.80	115.4	19.9	2.5
K**	100	20	15	4	6	5	3	2				2.45	5.5		

I/P* - intraperitoneal vaccination
K** - control

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 group, a significant increase in the titer of agglutinating antibodies was achieved within four weeks.

The GMT in the vaccinated group ranged upwards from 1:5.45, which was an eight fold increase of antibody titer in relation to the control.

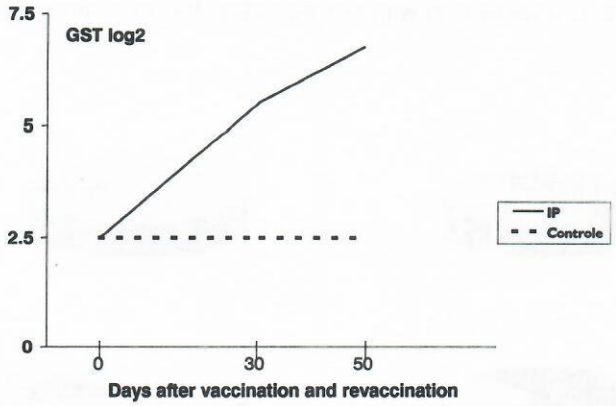

Figure 1. Classic agglutination test in fish after vaccination and revaccination

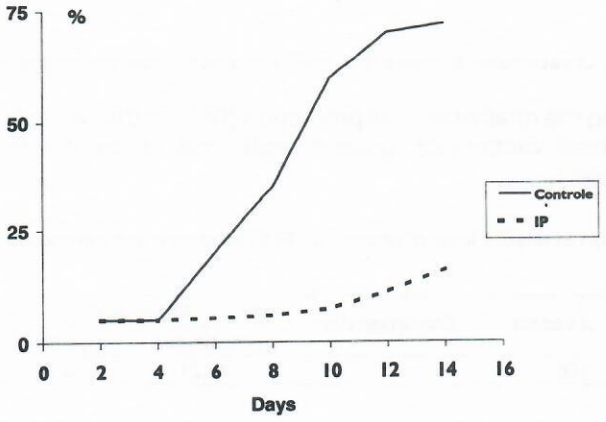

Figure 2. Mortality of vaccinated and control fish up to 14 days after challenge

Unvaccinated fish had greater mortality than vaccinated ones. The percentage was 73.11% in the control group, but 15.21% in the vaccinated group 14 days after challenge, (Table 3).

The dynamics of mortality are shown in figure 2.

Table 3. Mortality of vaccinated and revaccinated fish and controls 14 days after challenge

Treatment	Number of fish	Total mortality and % of mortality		Mortality and % of mortality from furunculosis	
		number	%	number	%
I/P*	92	24	26.08	14	15.21
K**	93	68	73.11	68	73.11

I/P* - intraperitoneal vaccination
K** - control

Each fish was infected i/p with 0.1 ml 320 x 10^8/ml culture of Aeromonas salmonicida.

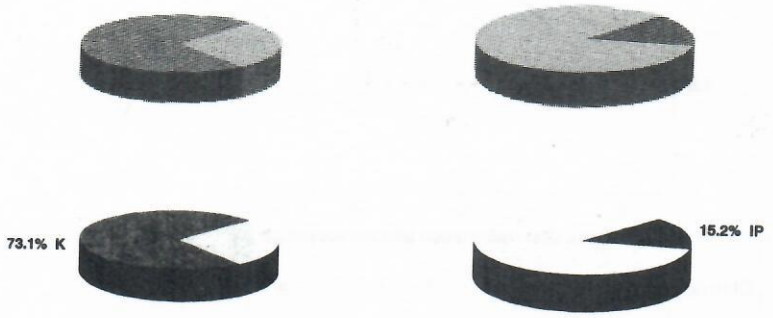

Figure 3. Mortality of vaccinated fish, and fish in control group 14 days after challenge

Comparing the relative level of protection (RLP) in the two groups, we found that intraperitoneal vaccination gave a high level of protection (79.20%) as indicated in Table 4.

Table 4. Comparison of relative level of protection (RLP) in control and intraperitoneally vaccinated groups

Route	No. of vaccin.	Challenge day	Mortality (%)		RLP
			Vaccinated	Control	
I/P	100	45	15.21	73.11	79.28

Four months after intraperitoneal vaccination of mature specimens a high level of circulating antibodies was recorded with the geometric mean titer of 1:7, vis-á-vis a control group value of 1:3.3. Thus, interaperitoneal vaccination of mature specimens led to a 13-fold increase in antibody titer in relation to the control group (Table 5.)

Table 5. Postvaccination immunity obtained by the method of classical agglutination 4 months after intraperitoneal vaccination of mature rainbow trout

Treatment method	No. of trout in group	No. of tested trout	Titer distribution Log^2							GMT Log^2	Anti Log	Titer enhancement in relation to control
			2	3	4	5	6	7	8			
I/P	100	20				3	3	5	9	7	128	13
K	200	20	3	10	5	2				3.3	9.8	

Ten days after spawning clinical symptoms of furunculosis due to natural attack of the disease appeared in the control group. In a short time the disease afflicted nearly 50% of the control fish. Therapy with antibiotics and a mineral

vitamin cocktail was conducted through out the entire duration of the disease. In spite of conscientiously conducted therapy, 20 mature fish died (10% mortality). Not one fish of the vaccinated group contracted the disease.

DISCUSSION

A significant degree of protection against furunculosis is achieved with intraperitoneal administration of adjuvanted vaccine in rainbow throat. According to Fijan et al. (1973), McCartny (1983), Evelin (1984), and Adams et al. (1988), the strongest immunological response is obtained by the intraperitoneal application.

Our results likewise clearly indicate that good 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 and Fryer (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.

We also found that the geometric mean titer of antibodies four months after intraperitoneal vaccination in mature rainbow trout was 13 times higher than in the control group. Furunculosis was not detected in mature fish during the spawning period. Vaccination conducted at the end of summer protected mature fish from natural infection during and after the spawning period.

The obtained results are support the conclusions of Hara et al. (1976) and Udey and Fryer (1987) that a single intraperitoneal application of inactivated bacterial cells of A. salmonicida in the spring achieves a high level of protection due to the large percentage of spawning stock survival (70–80%) after the spawning period when furunculosis appears.

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. These authors found no 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 natural infection than in the control.

For protection against artificial infection with the pathogenic species Aeromonas salmonicida intraperitoneal vaccination proved to be very effective since it protected 73,91% of the vaccinated fish. The control unvaccinated fish had a high percentage of mortality (73,11%).

From thus, we can conclude that intraperitoneal vaccination provided a significantly higher antibody titer and a significantly lower percentage of death than in the control unvaccinated groups.

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PRIPREMA VAKCINE ZA INTRAPERITONEALNU APLIKACIJU PROTIV FURUNKOLOZE KALIFORNIJSKIH PASTRMKI

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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 i kultivisanih na triptoza soja agaru (TSA).

Ispitivan je uticaj I/P vakcinacije na serološki odgovor vakcinisane kalifornijske pastrmke, te protektivni efekat primenjene vakcine nakon prirodne i veštački izazvane infekcije.

Zaključak je da I/P vakcinacija riba doprinosi dobrom serološkom odgovoru i da štiti vakcinisanu ribu od izlaganja patogenom terenskom izolatu bakterije A salmonicida.