Efficacy of Direct Immersion Vaccination of Ayu *Plecoglossus altivelis* with *Vibrio anguillarum* Bacterin

Katsuhiko KAWANO¹⁾, Takashi AOKI, and Tadatoshi KITAO

Department of Fisheries, Faculty of Agriculture, Miyazaki University
Miyazaki 880, Japan

(Received September 22, 1983)

There was no mortality in ayu (*Plecoglossus altivelis*) which had mean body weight of 4.2 or 9.2 g during the rearing period after direct immersion in underground water containing lyophilized formalin-killed cells or sonicated cells of *Vibrio anguillarum*. A high level of protection against vibriosis was found in fish immersed directly in 0.94 or 9.4 (wet)g/l lyophilized or sonicated bacterin. The efficacy was no different for fish with a mean body weight of 4.2 g immersed for 1, 3, or 10 min in 1 (wet)g/l lyophilized bacterin. The duration of protective immunity in fish with a mean body weight of 8.0 g lasted 60 days after direct immersion in 0.94 or 9.4 (wet)g/l of lyophilized bacterin.

Introduction

Vaccination by a direct immersion method in which salmonid fish were dipped into a non-hyperosmotic vaccine solution has been shown to result in excellent protection (GOULD *et al.*, 1979; EGIDIUS and ANDERSON, 1979). A high level of protection against vibriosis was provided to ayu (*Plecoglossus altivelis*) following immersion vaccination with a hyperosmotic solution containing lyophilized formalin-killed cells of *Vibrio anguillarum* (AOKI *et al.*, 1983). The duration of the protective response was demonstrated to be 113 days. Vaccines using lyophilized and washed cells of *V. anguillarum* were demonstrated to be safe for use with mature ayu (KAWANO *et al.*, 1984); however, vaccination using hyperosmotic solutions caused high mortality in young ayu. The direct immersion vaccination method should be safe and effective for use with ayu fry.

The onset of immunocompetence in ayu fry has not yet been determined and must be determined to successfully vaccinate cultured

ayu.

In the present study, we investigated the protection conferred on ayu fry by direct immersion vaccination with lyophilized or sonicated cells of *V. anguillarum*. The safety of the method and the duration of protective immunity were demonstrated for ayu fry.

Materials and Methods

The first experiment evaluated long immersion periods for fish in a lyophilized polyvalent vaccine containing formalin-killed cells of *V. anguillarum* PT24 (serotype A), PT496 (serotype B), and PT213 (serotype C) (supplied by Kitasato Institute) (KITAO *et al.*, 1983). Four groups of 100 ayu (mean body weight 8.0 g) were taken from a tank of fish collected from the freshwater Lake-Biwa. One group was immersed for 4 hrs in a bacterin concentration of lyophilized *V. anguillarum* cells equivalent to 9.4 (wet weight) g per liter (wet 1 g: about 10^9 cells). A second group was immersed for 4 hrs in a bacterin concentration of lyophilized *V. anguillarum* cells equivalent to 0.94 (wet) g per liter. A third group was immersed in the same 0.94 (wet) g/l level for 8 hrs. The fourth group was maintained as an unvaccinated control. These groups were maintained in separate tanks and fed crumbled pellets daily until challenge.

¹⁾ Present address: Central Laboratories, Kyoritsu Shoji Co. Ltd., Kukizaki-gun, Ibaragi Pref. 300-12, Japan.

Correspondence: T. AOKI, Department of Fisheries, Faculty of Agriculture, Miyazaki University, Miyazaki 880, Japan.

Lots of 20 fish each were removed from each treatment group 20, 30, and 60 days post vaccination and challenged with two levels of *V. anguillarum* SG7754 (serotype A) by the water-borne method (KAWANO *et al.*, 1983). Challenge was made by first exposing the fish for 2 min in a 5.32% NaCl solution and then in a physiological saline solution containing 10^5 or 10^6 cells for 3 min. The challenged fish were held at 20°C and observed daily for 10 days after challenge.

The mortality rate was determined for each group and the relative of percentages survival (RPS) (AMEND, 1981) was calculated for each group of vaccines compared to control as follows:

$$RPS = \left\{ 1 - \frac{\% \text{ vaccine mortality}}{\% \text{ control mortality}} \right\} \times 100$$

An RPS of 60 or more is considered to give a protective immunity to vaccinated fish (JOHNSON *et al.*, 1982b).

The second experiment evaluated short immersion periods for fish in a lyophilized vaccine containing formalin-killed cells of *V. anguillarum* PT479 (serotype A) and also evaluated the safety of direct immersion for ayu fry. Four groups of 60 ayu fry, with a mean body weight 4.2 g, were used. The first group was immersed for 1 min to underground water containing 0.94 (wet) g/l of lyophilized bacterin. The second and third groups were immersed in the same concentration of bacterin for 3 and 10 min, respectively. The fourth group served as a control and had the same size non-treated fish. The vaccinated fish and the non-vaccinated control fish were reared in tanks supplied with underground water at 20°C and fed pellets daily until challenge. Lots of 20 fish were challenged 15 days post vaccination. The challenge method was the same as in the first experiment; however, the challenge solution contained 10^4 or 10^5 viable cells/ml of *V. anguillarum*. Mortality in the challenged fish was observed 10 days after challenge. The mortality rate for the vaccinated lots was compared to that of the control lot to demonstrated the amount of protection.

The third experiment evaluated the protec-

tion for fish after vaccination by immersion with sonicated cells of *V. anguillarum* JA7011 (serotype A). The concentration of sonicated cells was equivalent to the number of viable 1.0×10^8 cells/ml. The first group with a mean body weight of 4.2 g and the second group, with a mean body weight of 9.2 g, were immersed for 3 min in underground water containing sonicated cells. The fish which had the same size were used as a control. The rearing period until challenge and method of challenge followed those of the second experiment.

Results

During the rearing period before challenging, there were no mortality in either vaccinated or control fish. This demonstrated that vaccination of ayu fry by direct immersion to lyophilized formalin-killed and sonicated cells of *V. anguillarum* did not adversely affect survival.

In the control group 20 days after beginning the experiment, all fish (mean body weight: 8.0 g) died when exposed to 10^5 or 10^6 cells/l of *V. anguillarum* (Table 1). On the other hand, no mortalities were recorded in the challenged fish which had been vaccinated by immersion for 4 hrs with 9.4 (wet) g/l lyophilized bacterin. The RPS were also observed to be more than 60 in fish challenged with 10^5 or 10^6 cells/l of *V. anguillarum*. The mortality rates in fish immersed for 4 or 8 hrs in 0.94 (wet) g/l 20 days post vaccination were significantly lower than those of control fish after challenge (Table 1).

The protective immunity in fish immersed for 4 hrs in 9.4 (wet)g/l lyophilized bacterin was not below 60 RPS 40 days post vaccination. Protection was also afforded fish immersed for 4 or 8 hrs in 0.94 (wet) g/l lyophilized cells.

The protective immunity in fish immersed for 4 hrs in 9.4 (wet)g/l lyophilized cells was below 60 RPS 60 days post vaccination after challenging with 10^5 cells of *V. anguillarum*. Strangely, the RPS in the same lot challenged by 10^6 cells/ml was than 60. The duration of protective immunity was maintained for these 60 days in fish immersed in 0.94 (wet)g/l

Table 1. Duration of protective immunity in fish vaccinated with direct immersion

Vaccination method		Challenge level Cells/ml	Days after immunization		
Concentration of vaccine solution	dipping time (hr)		20	40	60
9.4 (wet)g/l	4	10 ⁵	0* ¹ (100)* ²	0 (100)	30 (0)
		10 ⁶	30 (70)	10 (88)	20 (71)
0.94 (wet)g/l	4	10 ⁵	20 (80)	0 (100)	10 (77)
		10 ⁶	40 (60)	20 (75)	20 (71)
0.94 (wet)g/l	8	10 ⁵	20 (80)	0 (100)	10 (77)
		10 ⁶	30 (70)	0 (100)	30 (57)
Control	—	10 ⁵	100	20	30
		10 ⁶	100	80	70

*¹ Mortality rate.*² RPS: Relative of percentages survival defined as: $\left\{1 - \frac{\% \text{vaccinate mortality}}{\% \text{control mortality}}\right\} \times 100$.

Mean body weight of fish was 8.0 g at immersion and 17.1 g 60 days after vaccination. Mortality for challenged fish was observed 10 days after challenge.

Table 2. Efficacy of direct immersion in lyophilized formalin-killed bacterin of *V. anguillarum*

Vaccination method	Dipping time (min)	Challenge level (cells/ml)	Number of challenged fish	Number of survivors	Mortality rate (%)	RPS*
Immersion in 1 (wet)g/l of lyophilized bacterin	1	10 ⁴	20	20	0	100
		10 ⁵	20	14	30	50
	3	10 ⁴	20	20	0	100
		10 ⁵	20	10	50	17
	10	10 ⁴	20	20	0	100
		10 ⁵	20	18	10	83
Control	—	10 ⁴	20	12	40	
		10 ⁵	20	8	60	

* RPS: Relative of percentages survival.

Fish used had a mean body weight of 4.2 g.

The immersed fish were challenged 15 days after vaccination. Mortality for challenged fish was observed 10 days after challenge.

bacterin for 4 hrs or 8 hrs.

In the second experiment, protection for vaccinated groups compared to the control was demonstrated in fish (mean body weight; 4.2 g) immersed for 1, 3, or 10 min in 1 (wet) g/l of lyophilized cells of *V. anguillarum* 15 days post vaccination (Table 2). After challenging with 10⁴ cells of *V. anguillarum*, no differences in the level of protective immunity were observed in fish which had been immersed in the vaccine for 1, 3, or 10 min. On the other hand, the protective immunity in fish immersed for only 1 or 3 min was below 60 RPS,

after challenging with 10⁵ cells of *V. anguillarum*.

In the third experiment, the mortality rates for challenged fish of mean body weight of 4.2 g and 9.2 g which had been vaccinated by immersion with sonicated bacterin are shown in Table 3. Protective immunity in immersed fish at a mean body weight of 4.2 g was not below 60 RPS 15 days post vaccination. High level protection was also provided in fish of a mean body weight of 9.2 g immunized with sonicated bacterin.

Table 3. Efficacy of direct immersion with sonicated bacterin of *V. anguillarum*

Vaccination method	Dipping time (min)	Mean body weight	Challenge level (cells/ml)	Number of challenged fish	Number of survivors	Mortality rate (%)	RPS* ¹
Immersion in sonicated bacterin* ²	3	4.2	10 ⁴	20	20	0	100
			10 ⁵	20	17	15	75
Control		4.2	10 ⁴	20	12	40	
			10 ⁵	20	8	60	
Immersion in sonicated bacterin	3	9.2	10 ⁴	10	10	0	100
			10 ⁵	10	9	10	90
Control		9.2	10 ⁴	10	8	20	
			10 ⁵	10	0	100	

*¹ RPS: Relative of percentages survival.

*² The concentration of sonicated bacterin was equivalent to the number of viable cells 1.0×10^8 cells/ml. The immersed fish were challenged 15 days after vaccination. Mortality for challenged fish was observed 10 days after challenge.

Discussion

There were no mortality in fish directly immersed in lyophilized formalin-killed bacterin or sonicated bacterin of *V. anguillarum* during the rearing period after vaccination. We have previously observed a high mortality in fish of body weight of 5.4 g dipped in a hyperosmotic solution of NaCl and then in a vaccine solution of formalinized broth culture of *V. anguillarum* 3 days after treatment (AOKI *et al.*, 1982).

The ayu with a body weight of 4.2 g immunized by direct immersion were provided a high level of protection against vibriosis. The protection was also observed in fish directly immersed in a sonicated vaccine solution. ANTIPA *et al.* (1980) reported that the hyperosmotic infiltration method was slightly superior to direct immersion for protection with highly diluted bacterin. However, the protection was not significantly different in fish vaccinated by either method using undiluted and lowly diluted vaccine. JOHNSON (1980) also recognized the same efficacy in fish vaccinated by hyperosmotic infiltration and direct methods using vibrio vaccine. Our results concur that the step of dipping in a hyperosmotic solution is not necessary for vaccination against vibriosis. The direct immersion method we developed

using lyophilized formalin-killed or sonicated vaccine indicates that ayu fry can be immunized safely.

There was no difference in protection between direct immersion of ayu in 9.4 (wet) g/l or in 0.94 (wet) g/l lyophilized bacterin. This result shows that a 0.94 (wet) g/l concentration of bacterin in an immersed solution was adequate to provide protection against vibriosis in ayu.

In the first experiment, the fish immersed for 4 or 8 hrs in lyophilized bacterin showed a high level of protection against vibriosis. The immersion period was longer, because we had not yet obtained the results from the second experiment that showed that protective immunity was provided in fish immersed for only 1 min in a bacterin solution. JOHNSON *et al.* (1982a) found that significant improvement in the protection of sockeye salmon (*Oncorhynchus nerka*) occurred after a five sec. immersion time in bacterin. In direct immersion of ayu against vibriosis, the immersion period can be shortened to not more than 1 min in bacterin solution.

The duration of protective immunity found in ayu immersed in 5.32% NaCl solution containing lyophilized vaccine of *V. anguillarum* lasts for 113 days post vaccination (KAWANO *et al.*, 1984). In these experi-

ments the demonstrated immunological duration of 60 days was sufficient to provide immunity in ayu post direct immersion. There was no difference in efficacy for ayu immunized by the hyperosmotic infiltration method and the direct immersion method for 60 days post vaccination. We did not bother to check for protection in directly immersed fish more than 60 days after vaccination. Protective immunity is already thought to continue longer in directly immersed fish.

It is very interested to note the immuno-competence of young fish after vaccination. Chinnok salmon (*O. tshawytscha*) with an average size of 0.8 g responded to a *Vibrio* bacterin by oral vaccination (FRYER *et al.*, 1978). PATERSON *et al.* (1974) found that significant antibody titers were provided to coho salmon (*O. kisutch*) of 1.2 g by an injection of *A. salmonicida* bacterin. The onset of protective immunity was appeared in 1 g or larger salmonid fish after immersion in *V. anguillarum* (JOHNSON *et al.*, 1982a). However, the onset of protective immunity has not yet been determined for young ayu after immersion vaccination. Our data show that ayu with a body weight of 4.2 g can be effectively immunized with *V. anguillarum* by the immersion method. Further research is needed on the onset of protective immunity by the immersion method in ayu of less than 4.2 g.

Acknowledgements

We wish to thank Dr. Sei Takahashi of Shiga Prefectural Fisheries Experimental Station for his professional and technical help. Shiga Prefectural Fisheries Experimental Station kindly allowed us the use of their facilities and expertise. This research was supported in part by a grant-in-aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan.

References

- AMEND, D. F. (1981): Potency testing of fish vaccines. *Develop. Biol. Standard.*, **49**, 477-454.
- ANTIPA, R., R. GOULD and D. F. AMEND (1980): *Vibrio anguillarum* vaccination of sockeye salmon *Oncorhynchus nerka* (Walbaum) by direct and hyperosmotic immersion. *J. Fish Disease.*, **3**, 161-165.
- AOKI, T., T. KITAO, M. FUKUDOME, S. TAKAHASHI and S. EGUSA (1982): Immunization and challenge of ayu (*Plecoglossus altivelis*) against vibriosis by the hyperosmotic infiltration method. *Bull. Fac. Agri. Miyazaki Univ.*, **29**, 77-85.
- AOKI, T., T. KITAO, M. FUKUDOME, S. TAKAHASHI and E. EGUSA (1984): Modification of the hyperosmotic infiltration method in cultured ayu, *Plecoglossus altivelis* Temminck and Schlegel, against vibriosis. *J. Fish Disease.*, **7**, 149-156.
- EGIDIUS, E. C. and K. ANDERSEN (1979): Bath-immunization—a practical and non-stressing method of vaccinating sea farmed rainbow trout *Salmo gairdneri* Richardson against vibriosis. *J. Fish. Disease.*, **2**, 405-410.
- FRYER, J. L., J. S. ROHOVEC and R. L. GARRISON (1978): Immunization of salmonids for control of vibriosis. *Marine Fish. Rev.*, **40**, 20-23.
- GOULD, R. W., R. ANTIPA and D. F. AMEND (1979): Immersion vaccination of sockeye salmon (*Oncorhynchus nerka*) with two pathogenic strains of *Vibrio anguillarum*. *J. Fish. Res. Board Can.*, **36**, 222-225.
- JOHNSON, K. A. (1980): Development of effective vibrio vaccination program for salmonid culture. *Fish. Pathol.*, **14**, 181.
- JOHNSON, K. A., J. K. FLYNN and D. F. AMEND (1982a): Onset of immunity in salmonid fry vaccinated by direct immersion in *Vibrio anguillarum* and *Yersinia ruckeri* bacterins. *J. Fish disease.*, **5**, 197-205.
- JOHNSON, K. A., J. K. FLYNN and D. F. AMEND (1982b): Duration of immunity in salmonids vaccinated by direct immersion with *Yersinia ruckeri* and *Vibrio anguillarum* bacterins. *J. Fish Disease.*, **5**, 207-213.
- KAWANO, K., T. AOKI and T. KITAO (1983): Immersion vaccination and water-borne challenge of ayu (*Plecoglossus altivelis*) against vibriosis. *Fish Pathol.*, **18**, 143-149.
- KAWANO, K., T. AOKI and T. KITAO (1984): Duration of protection against vibriosis in ayu (*Plecoglossus altivelis*) vaccinated by immersion and oral administration with *Vibrio anguillarum*. *Bull. Japan. Soci. Scienti. Fish.*, **50** (in press).
- KITAO, T., T. AOKI, M. FUKUDOME, K. KAWANO, Y. WADA and Y. MIZUNO (1983): Serotyping of *Vibrio anguillarum* isolated from diseased

freshwater fish in Japan. *J. Fish Disease.*, 6, 175-181.

PATERSON, W. D. and J. L. FRYER (1974): Immune response of juvenile coho salmon (*Oncor-*

hynchus kisutch) to *Aeromonas salmonicida* cells administered intraperitoneally in Freund's complete adjuvant. *J. Fish. Res. Board Can.*, 31, 1751-1755.

アユのビブリオ病における直接浸せきワクチン法による防御免疫効果

河野勝彦*・青木 宙*・北尾忠利*

(昭和 58 年 9 月 22 日受理)

平均魚体重 4.2, 8.0 あるいは 9.2 g のアユを *Vibrio anguillarum* のホルマリン死菌凍結乾燥ワクチン (湿重量 0.94 g/l あるいは 9.4 g/l の濃度) あるいは超音波処理ワクチンを含む地下水中で浸せきを行なった。両ワクチンはこれらのアユに対してまったく毒性がなく、攻撃時までの飼育期間中斃死はなかった。ビブリオ菌の攻撃後、これらワクチン魚はビブリオ菌に対し防御反応を示した。さらに、平均魚体重 4.2 g のアユをホルマリン死菌凍結乾燥ワクチン 1 g/l の濃度で 1, 3 あるいは 10 分間浸せきを行ない、10 日後攻撃試験を行なったところ、いずれの浸せき区のワクチン魚も防御反応を示した。ワクチン浸せき時間による防御免疫反応の差は認められなかった。

---

* 宮崎大学農学部水産増殖学科