餌料生物を用いたアユのビブリオ病ワクチンの投与法

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Plankton-Mediated Oral Delivery of *Vibrio anguillarum* Vaccine to Juvenile Ayu

Kenji Kawai,*1 Shozo Yamamoto,*2 and Riichi Kusuda*1

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Juvenile ayu *Plecoglossus altivelis* are very sensitive to handling stress. For this reason, it is difficult to immunize the fish using immersion or spray delivery methods. In order to immunize these juvenile ayu against *Vibrio anguillarum* infection, oral delivery was attempted using feed plankton as a mediator of the vaccine. Two zooplankton species: water flea *Moina macrocopa* and brackish water rotifer *Branchionus plicatilis* were examined on their intake of the bacterial antigen. The water flea incorporated three kinds of *V. anguillarum* antigens: formalin-killed cells (FKC), formalin-killed and washed cells (FWC) and heat-killed cells (HKC), but the rotifer incorporated only HKC. One g of wet live water flea incorporated at most 520 μg lipopolysaccharide (LPS) of FKC for 30 min when FKC was added at a concentration of 1,400 μg wet FKC/10 water fea/m*l* culture water. The rotifer lost HKC easily by a few washings. Seventy-three-day-old juvenile ayu weighing an average of 63 mg incorporated 0.64 μg LPS (81 μg wet FKC)/fish in 2 h after FKC-fed water flea was given. Both the vaccinated fish by feeding FKC-fed water flea for 22 days and the unvaccinated fish fed untreated water flea were challenged with *V. anguillarum* by water-borne infection. The vaccinated and unvaccinated fish showed 92.4% and 64.2% survival rate, respectively, and this result indicates juvenile ayu efficiently incorporated the antigen and acquired protective immunity against vibriosis.

Vibriosis caused by *Vibrio anguillarum* is a serious disease in production farms and hatcheries of ayu *Plecoglossus altivelis* in Japan. Vaccination against this disease has been demonstrated to be effective using three kinds of delivery methods: oral administration,[1] immersion[2] and spray.[3] As juvenile ayu are so delicate that handling, net treatment and even careful transfer using pails damage them esily, hatched fish are usually cultured in the same pond until they grow to be about 1 g of body weight. Such stress sensitivity makes it difficult to immunize juvenile ayu by immersion or spray delivery methods. Oral administration is considered to be the safest method to immunize fish because it gives the least stress to fish. However this method is not applicable to postlarval and juvenile fish even if they are fed commercial powder or flake feed. These feeds are specially made to maintain floating property in the water. If they are soaked in antigen solution they loose their original buoyancy.

In the early steps of reproduction process, postlarval and juvenile ayu are fed live zooplankton which are also produced in the hatcheries. A brackish water rotifer *Branchionus plicatilis*[4] and a water flea *Moina macrocopa*[5] are used as the primary and secondary food organisms for ayu production. As it had been reported that these zooplankton consume bacteria as much as phytoplankton,[6] it was assumed that zoolplankton fed with a killed bacterial pathogen could be applicable to administer vaccine to juvenile ayu. In this study, it was examined how much the above zooplankton species incorporate antigen of the vaccine and if the juvenile ayu would acquire protective immunity by feeding vaccine-fed plankton.

Materials and Methods

Fish and Zooplankton

Seventy-day-old ayu which had been acclimatized to fresh water were transferred from production culture pond of Kochi Prefectural Freshwater Fisheries Laboratory to 180 *l* aquariums. Despite that the fish were transferred carefully using pails at night, about two thirds of the fish died by this treatment. The remaining fish were reared in fresh water at 16.5–18°C feeding *M. macrocopa*

*1 Fish Disease Laboratory, Faculty of Agriculture, Kochi University, Nankoku, Kochi 783, Japan (川合研児, 楠田理一: 高知大学農学部水族病理学講座).

*2 Okayama Prefectural Fisheries Experimental Station, Kashino, Ushimado, Oku, Okayama 701–43, Japan (山本章造: 岡山県水産試験場).

every day. When the fish became seventy-three-day-old, their mean body weight and total length were 63 mg and 27.3 mm respectively, they were used for vaccination.

Moina macrocopa and *B. plicatilis* were supplied from production culture ponds of Kochi Prefectural Freshwater Fisheries Laboratory.

Vaccines

Vibrio anguillarum strain V-36 serotype J-O-1,[7] which was originally isolated from a diseased ayu cultured in a fresh water pond and stocked by subculture, was cultured using nutrient broth containing 2% sodium chloride at 25°C without shaking for 2 days. Two thirds of the broth culture was inactivated with 0.5% formalin at 20°C for 2 days and the cell pellet harvested by centrifugation was used as formalin-killed cells (FKC). One half of FKC was washed with saline twice to eliminate formalin and used as formalin-killed and washed cells (FWC). One third of the broth culture was heated at 100°C for 30 min, washed with saline and used as heat-killed cells (HKC). The concentration of these inactivated bacterial wet cells was adjusted to 50% w/v in saline of 1.0% sodium chloride and stored until use.

Incorporation of Vaccines by Zooplankton

Seven hundred m*l* of the water flea culture at a concentration of about 10 individuals/m*l* in 1 *l* flask was added with the vaccine and incubated at room temperature (about 25°C) under different conditions. After incubation, the water flea were filtered with nylon plankton net, weighed, washed once with saline and used to determine the incorporated antigen.

To compare uptake rates of FKC, FWC and HKC, those vaccines were added to the water flea culture at concentrations of 700 μg or 1,400 μg wet weight of cells/m*l* and incubated for 1 or 3 h. To differentiate the amount of the antigen incorporated in the water flea from that adhering to it, effect of washing on the amount of the remaining antigen in the vaccine-fed water flea was examined. The water flea was incubated for 2 h in 700 m*l* FKC suspension at a concentration of 1,400 μg of FKC/m*l*. Aliquots of 0.30 g water flea were collected and washed various times with 5 m*l* saline, then the amount of the antigen in the water flea or the washing saline was determined. To compare incorportation efficiency at different concentrations of vaccine, water flea was incubated at concentrations of 350, 700, 1,400, 2,100, 2,800 and 3,500 μg of FKC/m*l* for 2 h. To examine change in the amount of incorporated antigen in the course of incubation time, water flea were incubated for 3 h in FKC suspension at a concentration of 1,400 μg of FKC/m*l*, then transferred into the water without FKC and incubated for three more hours. One hundred m*l* of the water flea culture was sampled every hour and the amount of the antigen incorporated in the plankter was determined.

The rotifer was incubated in about 50% sea water at a concentration range of 350–450 individuals/m*l* in the vaccine suspensions. Procedure to compare effects of different conditions on the incorporation of the vaccine was same to that used for the water flea except that HKC was used mainly.

Quantitative Determination of Antigen

After incubation with vaccines, 0.30 g of the water flea and rotifer were washed once with saline, homogenized with 5 m*l* saline using Teflon homogenizer, dispersed by Ultrasonic Distrupter UR-200P (Tomy Seiko) and centrifuged twice at 1,250×g for 20 min. Amount of the antigen in the supernatant was determined by passive hemagglutination inhibition test (PHAI) using anti-V-36 rabbit serum and sheep red blood cell (SRBC) sensitized with crude lipopolysaccharide (LPS) extracted from *V. anguillarum* V-36 by Westphal's phenol-water procedure[8] without a step of ultracentrifugation. Lyophilized LPS was dissolved in physiological saline at a concentration of 1,000 μg/m*l*. One tenth m*l* of washed SRBC was added into 1 m*l* of LPS solution and incubated at 37°C for 1 h. Sensitized SRBC was washed and resupended in physiological saline of 20 m*l*. Agglutination titer of the rabbit antiserum to the sensitized SRBC was determined first by the microtiter method. The highest dilution that showed agglutination was determined as one unit. Serial two fold dilutions of 25 μ*l* of specimen and standard solution of crude LPS or sonicated FKC were added with 25 μ*l* of four unit antiserum dilutions, incubated at 25°C for 2 h then at 4°C overnight. To the mixtures of antigen and antiserum, 50 μ*l* of sensitized SRBC was added and incubated at 25°C for 2 h then at 4°C overnight. The quantity of the antigen was determined by comparing the highest dilution of each specimen that showed negative agglutination to that of the standard dilution of LPS. The ratio of the weight of lyophilized LPS: wet FKC was determined to be 1:126 by comparing PHAI titer between

standard solution of LPS and sonicated wet FKC.

The water flea were incubated in FKC suspension at a concentration of 2,000 μg FKC/ml for 30 min, then given to a group of fish at a rate of 100 water flea/fish. Five fish were collected from the group before and 1, 2, 3, 4, 6 and 24 h after the FKC-fed water flea were given. The fish were washed with saline, pooled and stored at −40°C until further use. The amount of LPS incorporated in the ayu was determined by the same method used for zooplankton.

Vaccination and Challenge of Ayu

A group of about 100 fish was vaccinated by feeding FKC-fed water flea once a day for 22 days. Another goup of about 100 fish was given untreated water flea. Fish were challenged on the following day after the final vaccination was done when mean body weight and mean total length of the fish were 115 mg and 33.3 mm, respectively. Level of the rearing water was lowered to about one third and virulent *V. anguillarum* strain V-2039 of the same serotype as V-36 was added at a concentration of 8.5×10^6 cells/ml. Five min after exposure, water flow was resumed. To confirm the cause of mortalities, liver and kidney smears of the dead fish were stained with anti-V-36 fluorescent antibody to detect *V. anguillarum*.

Results

Comparison Among Vaccine Preparations

Comparison of the amount of the vaccine antigen incorporated in both plankters among different preparations is shown in Table 1. By incubating at a concentration of 700 μg of FKC, FWC and HKC/ml, the water flea incorporated 390, 260 and 130 μg LPS/g of wet weight of the water flea, respectively for 1 h and 260, 390 and 520 μg LPS/g, respectively for 3 h. At the concentration of 1,400 μg of FKC and FWC/ml, the water flea incorporated 520 and 260 μg LPS/g respectively for 1 h and 1,040 and 260 μg LPS/g respectively for 3 h.

The rotifer incorporated only HKC while FKC or FWC were not incorporated. When the rotifer were incubated at a concentration of 700 μg HKC/ml for 1 and 3 h, amounts of the incorporated antigen were 130 and 260 μg LPS/g of wet weight of the rotifer, respectively. When incubated at a concentration of 1,400 μg HKC/ml for 1 and 3 h, they contained 260 and 520 μg LPS/g, respectively.

Effect of Washing on Plankton-associated Antigen

Fig. 1 shows the change in the amount of antigen associated with both plankters after washing. The amount of the antigen detected from the water flea were 1,560, 780, 520 and 260 μg LPS/g before and after the first, second and third washing, respectively. The concentration of the antigen in the water flea culture filtrate, the first, second and third washing saline were 31, 12, 12 and 4 μg LPS/ml, respectively.

The amount of antigen detected from the rotifer were 390, 260, 66 and <17 μg LPS/g before and after the first, second and third washing, respectively. The concentrations of antigen in the rotifer culture filtrate, the first, second and third washing saline were 16, 8, 6 and <1.0 μg LPS/ml, respectively. No antigen was detected in the rotifer or the washing saline after the third washing.

Effect of Vaccine Concentration

The amount of vaccine antigen incorporated in the zooplankton increased as the concentration of vaccine in the zooplankton culture was increased as shown in Fig. 2. The water flea incorporated 130, 260, 390, 520, 520 and 520 μg LPS/g of water flea when incubated at concentrations of 350, 700, 1,400, 2,100, 2,800 and 3,500 μg FKC/ml, respectively. The amount of antigen incorporated by

Table 1. Incorporation of three kinds of vaccines by *Moina macrocopa* and *Branchionus plicatilis*. The amount of incorporated antigen was expressed in μg LPS/g wet zooplankton

Concentration of vaccine (μg wet cell/ml)	Incubation period (h)	*M. macrocopa*			*B. plicatilis*		
		FKC	FWC	HKC	FKC	FWC	HKC
700	1	390	260	130	<17*	<17	130
	3	260	390	520	<17	<17	260
1,400	1	520	260	ND	<17	<17	260
	3	1,040	260	ND	<17	<17	520

The zooplankton were washed once with saline after incubation in the vaccine suspensions.
Abbreviations; FKC: formalin-killed cell, FWC: formalin-killed and washed cell, HKC: heat-killed cell, ND: not done.
* The limit of detection was 17 μg LPS/g.

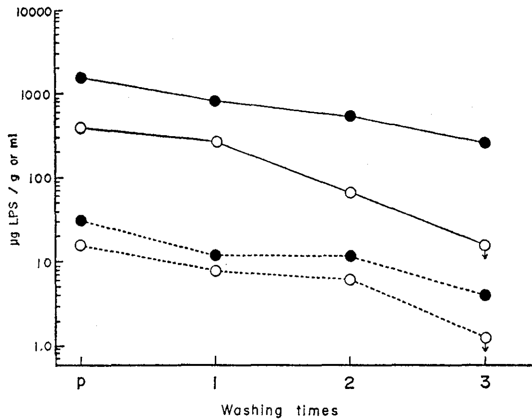

Fig. 1. Effect of washing on the amount of *Vibrio anguillarum* antigen remaining in *Moina macrocopa* and *Barnchionus plicatilis*. *Moina macrocopa* and *B. plicatilis* were incubated at concentrations of 1,400 μg/m*l* of FKC and HKC, respectively for 2 h then washed different times with saline. Antigen in the zooplankton, plankton culture filtrate and washing saline were quantitatively determined before and after washed once, twice and three times. Marks; ●: *M. macrocopa*, ○: *B. plicatilis*. Lines; ——, zooplankton, ---: culture filtrate or washing saline. Arrow: below the limit of detection (17 μg LPS/g wet zooplankton, or 1.0 μg LPS/m*l* washing saline).

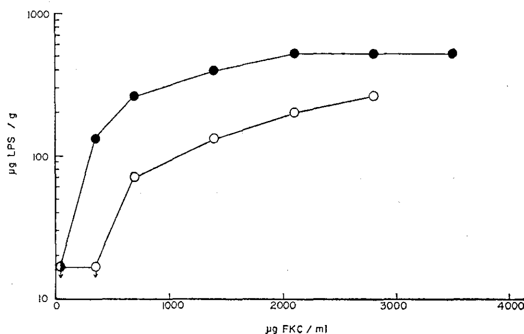

Fig. 2. Effect of vaccine concentration on the incorporation of antigen by *Moina macrocopa* and *Branchionus plicatilis*. *Moina macrocopa* and *B. plicatilis* were incubated in different concentrations of FKC and HKC, respectively for 2 h. Marks; ●: *M. macrocopa*, ○: *B. plicatilis*. Arrow: below the limit of detection (17 μg LPS/g wet zooplamkton).

the rotifer also increased as the concentration of vaccine in the rotifer culture increased. The rotifer incorporated 70, 130, 200 and 260 μg LPS/g of rotifer when incubated at vaccine concentrations of 700, 1,400, 2,100 and 2,800 μg HKC/m*l*, respectively. No antigen was detected in the rotifer when the vaccine concentration was 350 μg HKC/m*l*.

Change in the Amount of Antigen During Incubation

Change in the amount of the antigen in both plankters during and after the incubation with vaccine is shown in Fig. 3. The amount of antigen incorporated by the water flea were 520, 520, 780 and 520 μg LPS/g when incubated with FKC for 30 min, 1, 2 and 3 h, respectively. The re-

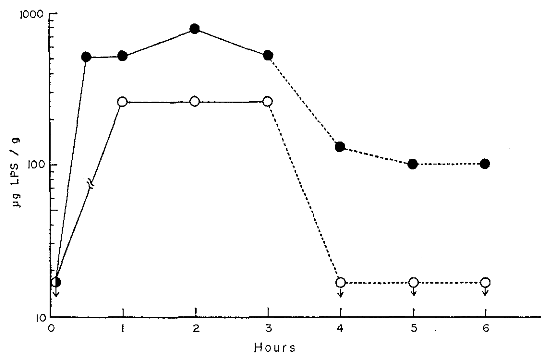

Fig. 3. Change in the amount of the antigen incorporated by *Moina macrocopa* and *Branchionus plicatilis* during and after the exposure to the vaccine. *Moina macrocopa* and *B. plicatilis* were incubated at a concentration of 1,400 μg/m*l* of FKC and HKC, respectively for 3 h then transferred to clear water. Marks; ●: *M. macrocopa*, ○: *B. plicatilis*. Lines; ——: incubated with vaccine, ---: incubated without vaccine. Arrow: below the limit of detection (17 μg LPS/g wet zooplankton).

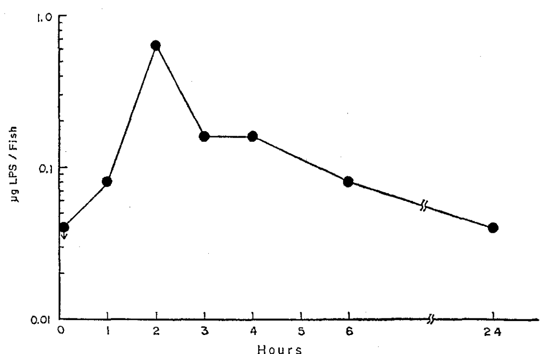

Fig. 4. Change in the amount of the antigen in ayu which were given vaccine-fed *Moina mcrocopa. Moina macrocopa* was incubated in FKC suspension at a concentration of 2,000 μg FKC/m*l* for 30 min then given to the fish at a concentration of 100 *M. macrocopa*/fish. Arrow: below the limit of detection (0.04 μg LPS/fish).

maining antigen in the water flea was 130, 100 and 100 μg LPS/g 1, 2 and 3 h after transferred into water without FKC, respectively.

The amount of incorporated antigen by the rotifer was 260 μg LPS/g throughout 3 h of incubation with HKC, but no antigen was detected after the plankter had been transferred into water without vaccine.

Incorporation of Antigen by Ayu

Change in the amount of incorporated antigen by ayu was shown in Fig. 4. Antigen was detected for 24 h after feeding started. Maxmium amount of the antigen was 0.64 μg LPS/fish which was determined after 2 h.

Protection of Vaccinated Ayu Against Challenge

Survival data of the challenged fish are shown in Table 2 and Fig. 5. Mortality occurred from 2 to 3 days after the challenge in the vaccinated group and from 2 to 4 days in the unvaccinated group. Decrease of the survival rate was remarkable in the unvaccinated fish. The final survival rates 8 days after challenge were 92.4% in the vaccinated fish and 64.2% in the unvaccinated fish. *Vibrio anguillarum* was detected in all the dead fish by fluorescent antibody techinque, which showed the mortality was brought about by *V. anguillarum* infection in the challenge test.

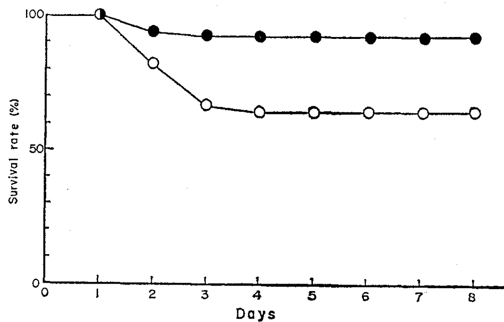

Fig. 5. Change in the survival rate of vaccinated and unvaccinated ayu after challenge. Marks; ●: vaccinated fish, ○: unvaccinated fish.

Table 2. Mortalities of vaccinated and unvaccinated ayu after challenge

	Number of fish			Survival rate (%)
	Used	Challenged	Dead	
Vaccinated	114	79	6	92.4
Unvaccinated	81	81	29	64.2

Discussion

In the reproductive hatcheries of ayu, *B. plicatilis* is the first food organism during the early stage of fish larvae until about 40 days after hatching when the body length is less than 20 mm. Later, *M. macrocopa* or brine shrimp *Artemia salina* are fed until 60–80 days after hatching when the body length is 25–30 mm. It was assumed that in this phase, vaccine administration can be conducted using *M. macrocopa* and *B. plicatilis* because it had been reported that they consume great amount of bacteria.[8] Yasuda and Taga examined which of the bacteria were efficiently consumed by *B. plicatilis*.[9] They showed that *Pseudomonas* spp. were useful for feed but *Vibrio* spp. were not suitable because the latter remained in the rotifer for 4 to 12 h suggesting that vibrios were not digested well. *Moina macrocopa* is bigger than *B. plicatilis* and it was also reported to feed on many species of bacteria.[8,10]

The water flea incorporated three kinds of vaccines but, the rotifer did not feed on formalin-treated cells even after washing to eliminate formalin. Microscopic observation of these vaccine supensions showed that bacterial cells of FKC and FWC aggregated into large particles so that the rotifer could not feed on them but such large particles were not observed in the HKC suspension. Yasuda and Taga showed that rotifers tend to feed on particles of 5–20 μm in diameter.[9] Hino and Hirano also showed that the optimal diameter of dried active sludge were 21.5 and 26.5 μm to feed rotifers of two sizes having 240 and 300 μm long lorica, respectively.[11] It is assumed that the reason why the rotifer did not incorporate FKC or FWC is that the treatment with formalin made the vaccine particles too large for the rotifer.

Vaccine-fed water flea and rotifer lost much of the antigen by washing probably beacuse some of the antigen associated only to the surface of the plankters. Such antigen is supposed to come off easily from them when introduced into the fish pond. Nevertheless, the water flea contained 30–100 mg of wet packed vaccine/g after washing once. The rotifer lost more antigen than water flea. This may be because the rotifer has incorporated less amount of antigen than that adhering to it or the rotifer is more fragile than the water flea for washing.

After single administration of water flea, juvenile ayu incorporated 0.64 μg LPS (81 μg wet FKC)/fish at most that was calculated to be about

10 mg LPS (1.3 g wet FKC)/kg of fish body weight. Kawai and Kusuda showed that immature and adult ayu were effectively immunized when they were administered 2.54 mg LPS/kg of fish body weight/day for 10 days[12] or 0.1 g FKC/kg of fish body weight/day for 8–14 days.[13] From these informations, it seems that the ayu in the present study should have incorporated sufficient amount of the antigen.

The challenge test showed that the vaccinated fish had significantly higher survival rate than the unvaccinated fish. It is concluded that protective immune response was induced in the juvenile ayu by feeding vaccine-fed *M. macrocopa*. The present study shows that the oral delivery method of *V. anguillarum* vaccine using food plankton is effective to prevent vibriosis in juvenile ayu in the very early stage of growth. As zooplankton is usually used for primary and secondary feed in the reproduction of many species of fish, the present method is considered to be applicable to immunize these fishes in hatchery management.

References

1) R. Kusuda, K. Kawai, Y. Jo, T. Akizuki, M. Fukunaga and N. Kotake: *Nippon Suisan Gakkaishi*, **44**, 21–25 (1978).
2) T. Aoki and T. Kitao: *Fish Pathol.*, **13**, 19–24 (1978).
3) T. Itami and R. Kusuda: *Nippon Suisan Gakkaishi*, **44**, 1413 (1978).
4) R. Ishida: in "Ayu", 8th ed., Midori Shobo, Tokyo, 1985, pp. 49–73.
5) N. Suzuki: in "Tansui Yoshoku Gijutu" (ed. by M. Nomura), Koseisha Koseikaku, Yokyo, 1982, pp. 254–267.
6) C. A. Stuart, M. McPherson, and H. J. Cooper: *Physiol. Zool.*, **4**, 87–100 (1931).
7) Y. Ezura, K. Tajima, M. Yoshimizu, and T. Kimura: *Fish Pathol.*, **14**, 167–179 (1980).
8) O. Westphal and K. Jann: in "Method in Carbohydrate Chemistry" (ed. by R. L. Whistler *et al.*), Vol. V, Academic Press, New York, 1965, pp. 83–91.
9) K. Yasuda and N. Taga: *Nippon Suisan Gakkaishi*, **46**, 933–939 (1980).
10) A. Shirota: Fresh-Marine Organism as Living Feed for Fisheries, Koseisha Koseikaku, Tokyo, 1975, pp. 110–111.
11) A. Hino and R. Hirano: *Nippon Suisan Gakkaishi*, **46**, 1217–1222 (1980).
12) K. Kawai and R. Kusuda: *Nippon Suisan Gakkaishi*, **49**. 511–514 (1983).
13) K. Kawai and R. Kusuda: *Fish Pathol.*, **20**, 413–419 (1985).

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