Immersion Immunization of Ayu (*Plecoglossus altivelis*) with *Pseudomonas plecoglossicida* Bacterin

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Bacterial hemorrhagic ascites (BHA) of ayu has been reported as a new disease causing mass mortality among cultured ayu (*Plecoglossus altivelis*)¹⁾. The causative agent of the disease was recently given the name *Pseudomonas plecoglossicida*²⁾. The disease is characterized by bloody ascites with a large number of bacteria in the blood and internal organs of affected fish.

As *P. plecoglossicida* is resistant to medicines licenced for cultured ayu, the development of a protective vaccine is a possible method for the control of BHA. In the present study, immersion immunization of ayu with formalin-killed *P. plecoglossicida* bacterin was evaluated by challenge test. The kinetics of invading *P. plecoglossicida* in the organs of immunized fish was also investigated.

Materials and Methods

Ayu weighing about 7 g were obtained from Yamanashi Prefectural Fisheries Technology Center and maintained at about 23°C at the Fisheries Laboratory of the University of Tokyo from July to September 1997.

P. plecoglossicida strain FPC 941 was isolated from an ayu during an outbreak of BHA at Shiga Prefecture in 1994. The strain was kept frozen at –80°C in tryptic soy broth (Nissui) containing 10% (v/v) glycerol.

The bacterin, composed of formalin-killed cells of *P. plecoglossicida* strain FPC 941, was provided by Kyouritsu Shouji Co. Ltd., Tokyo at a concentration of 1.7×10^{10} CFU/mL. The vaccine was diluted 1:100 in dechlorinated water shortly before use. Three hundred fish were taken from the stock, and divided into experimental and control groups. The experimental fish were immersed in 40 L of the vaccine solution for 30 min with aeration. Control fish were immersed in dechlorinated

water for 30 min. Two weeks after immunization, the experimental fish were divided into two groups, one of which was given a booster immersion. The booster was performed for 20 min in the same manner as the initial immunization. Water temperature during the experiment was about 23°C.

Immunized and control fish were challenged with *P. plecoglossicida* by immersion in 3×10^7 CFU/mL for 15 min at either 2, 4 or 6 wk after the initial immunization. Ten fish were transferred to a 30 L container with flowing water for observation of mortality, and the remaining fish were transferred to another container for observation of the fate of challenging bacteria in the liver, spleen, kidney and blood of the fish. Mortalities were recorded for 10 days post-challenge, and the efficacy of vaccination was evaluated as relative percent survival (RPS)³⁾.

Three fish were taken from each group, 1, 2 and 3 days after challenge with the bacteria. Fish were anesthetized with 250 ppm of 2-phenoxyethanol and then bled from the caudal vein with a heparinized syringe. The liver, spleen and kidney samples were separately homogenized (10% w/w) in sterile physiological saline. The blood and homogenates were serially diluted 10-fold with physiological saline. Duplicates of 25 µL of each diluted solution were plated on tryptic soy agar (Nissui). After 24 h of incubation at 25°C, bacterial colonies were counted, and colony forming units (CFU) per g of tissue or mL of blood was determined.

Data of mortalities were analyzed by Fisher's exact probability test. Differences were considered significant at $P < 0.05$.

Results and Discussion

In challenge tests with *P. plecoglossicida*, the immunized fish had lower mortalities than the controls (Table 1). Three days after challenge, both immunized and control fish began dying, and a symptom of BHA, bloody ascites, appeared in the dead fish. The mortalities continued for 1 wk. The results indicate that immersion immunization induces protective immunity against BHA in ayu. In addition, it is concluded that a booster immunization is effective in keeping protective immunity longer.

The fate of invading *P. plecoglossicida* in the organs of ayu 6 wk after immunization is shown in Table 2. In fish immunized twice, the bacterium was not detected at 1, 2 and 3 days post-challenge. A high level of protection against *P. plecoglossicida* infection was seen in this group. However, in the fish immunized once, the bacterium was detected in one of three fish at 1, 2 and 3 days post-challenge. We suppose that some fish did not have sufficient protective immunity. In the control fish, the bacterium was detected at 1 day post-challenge, and increased in number toward 2 and 3 days post-challenge. Similar results were obtained in the chal-

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Table 1. Duration of protection : mortalities of immunized and unimmunized control ayu 2, 4, and 6 wk after initial immunization with a *Pseudomonas plecoglossicida* bacterin

Weeks after initial vaccination	Fish group	Number of challenged fish	% Mortality	RPS	Fisher's exact probability
2	Immunized	10	70	22	0.29
	Unimmunized control	10	90		
4	Immunized twice [#]	10	60	40	0.04 [◆]
	Immunized once	10	60	40	0.04 [◆]
	Unimmunized control	10	100		
6	Immunized twice [#]	10	60	40	0.04 [◆]
	Immunized once	10	80	20	0.24
	Unimmunized control	10	100		

[#] : Booster was administered 2 wk after the initial immunization.

[◆] : Significantly different when compared with unimmunized control ($P < 0.05$)

Table 2. Distribution of *Pseudomonas plecoglossicida* in ayu challenged by immersion at a concentration of 3×10^7 CFU/mL 6 wk after initial immunization

Days after challenge	Group	<i>P. plecoglossicida</i> in (CFU/g or mL)			
		Liver	Spleen	Kidney	Blood
1	Immunized twice	—	—	—	—
	Immunized once	1.2×10^3	—	2.5×10^3	—
	Unimmunized control	—	—	4.6×10^3	—
		—	—	8.0×10^3	—
2	Immunized twice	—	—	—	—
	Immunized once	—	2.7×10^5	1.0×10^4	4.0×10^2
	Unimmunized control	—	—	8.0×10^2	—
		—	9.0×10^3	8.8×10^3	—
3	Immunized twice	—	—	—	—
	Immunized once	1.3×10^6	1.0×10^8	7.4×10^6	8.0×10^5
	Unimmunized control	2.0×10^3	9.0×10^4	9.8×10^3	—
		2.0×10^4	1.0×10^5	4.3×10^3	—
		2.4×10^4	2.3×10^5	5.4×10^4	2.6×10^2

—: not detected (less than the limit of detection, 4×10^2 CFU/g of tissues or 4×10^1 CFU/mL of blood)

lenge tests 2 and 4 wk after the initial immunization (data not shown). The high CFU level of *P. plecoglossicida* in control fish 3 days after challenge indicates a rapid proliferation of the bacteria in fish succumbing to infection.

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