Jurnal Perikanan UGM (GMU J. Fish. Sci.) I (2) : 1-9 ISSN : 0853 - 6384

VACCINATION WITH *VIBRIO ANGUILLARUM* AND *VIBRIO ORDALII* BACTERINS AGAINST VIBRIOSIS

Kamiso H.N [1]

Introduction

The potential importance of marine and brackishwater fish farming and serious problem caused by vibriosis have stimulated efforts toward the prevention and control the disease (Nash *et al.*, 1990; Lupiani *et al.*, 1993; Limsuwan, 1993). Under conditions of captivity, vibriosis may be controlled by careful husbandry practices that reduce stress, and rational use of antibiotics and immunization (Richards, 1980; Tabata *et al.*, 1982; Estevez *et al.*, 1994). However, it would not be recommended to use subtherapeutic levels of antibiotics for a long period of time because resistant straints of pathogenic bacteria could develop (Hahnel and Gould, 1982; Kerry *et al.*, 1994). In addition, antibiotics should have side effects on the fish including suppresive effects on the immune response (Grondel and Boesten, 1982; Lewis *et al.*, 1985).

There are different methods of vaccination to prevent the oubreak of vibriosis in cultured fish. These include : bath or direct immersion (Egidius and Andersen, 1984), oral bacterin incorparated with feed (Kawamoto *et al.*, 1984), spray (Itami and Kusuda, 1980), intraperitoneal or intra muscular injection (Amend and Johnson, 1984; Estavez, *et al.*, 1994), hyperosmotic infiltration (Aoki *et al.*, 1984), and passive immunization (Viele *et al.* , 1980; Aoki *et al.*, 1984).

The objectives of the study were to determine the immune response and duration of antibody titer in the sera of English sole and chum salmon after immunization either with *V. ordalli* or *V. anguillarum* bacterin.

Materials and Methods

Bacteria

Culture of *Vibrio anguillarum* (isolate LS 173) and *Vibrio ordalii* (isolate MSC 275) were provided by the Fish Disease Laboratory at the Hatfield Marine Science Center. Before the bacteria were used in experiments they were passed through chum salmon (*Oncorhynchus keta*) or English sole (*Parophrys vetulus*) three times by water-borne exposure.

Fish

Juvenile English sole (*P. vetulus*) were collected with a 5-m otter trawl in Yaguina Bay, Oregon. To remove ectoparasites, especially *Gyrodactylus* sp. that are very commonly found on their fins, the juvinile English sole were treated with a I : 4,000 formalin solution for one hour (Put and Hoffman, 1963). Salmon used in experiments were fingerling chum salmon (*O. keta*) obtained at Fish Hatchery on Netarts Bay. Both English sole and chum salmon were tested for vibrio antibody titers to determine if they had previous infections with *V. anguillarum* or *V. ordalii.*

Challenge method

Water-borne exposure to bacteria was used in most experiments. The procedure followed the method described by Gould (1977).

The fish were exposed to bacterial

[1] Department of Fisheries, Faculty of Agriculture, University of Gadjah Mada, Yogyakarta 55281, Indonesia.

suspension for 30 minutes with aeration. After 30 minutes water flow was restored to normal levels with flow rate between 2 and 3 liters per minute. All experiments using water-borne exposures were terminated in 14 days and fish were not fed.

Vibrio anguillarum and Vibrio ordalii bacterin preparation.

A 10-ml broth culture of each bacterium was prepared in *Trypticase Soy Broth* (TSB) and incubated at 18°C for 24 hours. Each of these cultures was then transferred into 250 ml TSB and incubated at 18°C on an agitator. After 24 hours each was transferred again into 1 liter TSB and incubated the purity was tested and the cells were harvested. Harvesting the bacterial cells from these broth cultures was accomplished with the aid of a Backman Model J2-21 centrifuge. The cells were washed three times in sterile phosphate-buffered saline (PBS, pH7.0) with centrifugation at 3.000 rpm for 10 minutes at 0°C. The harvested cells were resuspended in 40 ml sterile PBS containing 0.3% formaldehyde and kept in refrigerator for 24 hours. After 24 hours the cells were again washed and centrifuged three times (10 minutes, 3.000 rpm, at 0°C in sterile PBS). Vaccines were prepared by resuspension of the wet-packed cells in sterile PBS about 0.85 optical density at 525 nm using a spectrophotometer. Three 0.1 ml aliquots of the suspensions were inoculated into *Trypticase Soy Agar* (TSA) to test sterility. The absence of growth indicated non-viability of the bacterial cells. The vaccines were frozen until they were used (Rohovec *et al.*, 1981).

Harvesting Fish Sera.

Fish blood was collected by cutting the caudal peduncle. Individual or pooled blood samples were allowed to clot at room temperature for one hour and overnight at 4°C. The serum fraction was harvested by centrifugation (3.000 rpm, for 10 minut at 0°C). The serum was used immediat or frozen in a sterile tube until use.

Antigen preparations for microtiter

Antigens for microtiter tests were prepar by the same procedure as in vacci preparation except for formalin treatme The concentration of the antige suspensions were adjusted to an optic density of 0,85 at 525 nm using spectrophotometer and stored at 4°C ur they were used.

Microtiter technique

Fifty µl of serum was used in the microtit technique for determination of antibo titer. Serial doubling dilutions of the seru sample using PBS were made in a 96 w microtiter plate. After addition of antige (50 µl per well), the plate was shake gently for two minutes before incubatir two hours at room temperature ar overnight at 4°C. The antibody titer wa determined after incubation by observin the microtiter plates under a dissectin microscope.

Immune response experiment

There were 160 juveniles English sol (11.2 ± 0.9 cm, 7.4 ± 1.7 g) and 16 fingerlings chum salmon (15.8 ± 1.5 cm 36.0 ± 7.5 g) used in the first experiment Each fish species was divided into tw groups consisting of 80 fish each an maintained separately in circular tank containing 125 liters seawater supplied wit pathogen free seawater (2 to 3 l/min) a 14°C for 15 days. The fish were fed dail with moist pellets, about 5% of body weigh for English sole, and about 3% for chum salmon. After 15 days, the first group o species was immunized by intra-peritoneal injection with 0.1 ml (of 10^9 cells/ml) of formalin killed *V. ordalii* bacteria using 1.0 ml tuberculin syringe and 26 G x 2 inch needle. The second group of each species

was injected with 0.1 ml sterile PBS (pH 7.0) and used as a control.

Tricaine methane sulfonate (MS 222), 50 mg/l was used as an anesthetic while the fish were being injected. The second experiment was identical to the first except that *V. anguillarum* was source of the bacterin.

Each week 2 or 3 fish were sampled from each tank to check the immune response. The microtiter method was used to determine the antibody titers of fish from each tank. When the antibody titers of immunized fish had reached a peak, about 4 weeks post immunization, the fish were challenged with live *V. ordalii* or *V. anguillarum*. Forty fish were taken randomly from each tank and divided into four groups (10 per group). The first two groups were challenged with the bacteria that had been passed through English sole, and the other two groups were challenged with the bacteria that had been passed through chum salmon. Waterborne exposure was used for this purpose, and conducted under conditions previosly described. Mortality was checked daily and bacterial pathogens were isolated and identified as described earlier. The relative percent survival (RPS) was calculated using the formula adapted by Johnson *et al.*(1982a).

$$RPS = \left(1 - \frac{\%\ \text{mortality of vaccinated fish}}{\%\ \text{mortality of unvaccinated fish}}\right) \times 100\%$$

The remainder of the fish in each tank was sampled weekly until the eighth week, and monthly after eight weeks, to determine the duration of immune response.

Rerults

Immune response experiments.

The mortality rate of English sole challenged with *V. ordalii* ranged from 10 to 20% in vaccinated fish, and from 30 to 70% in unvaccinated fish. In English sole that were challenged with *V. anguillarum* the mortality rate ranged from 10 to 20% in vaccinated fish, and from 40 to 60% unvaccinated fish. There was no mortality in vaccinated chum salmon challenged with *V. ordalii*, and only one out of four replicates had a 10% mortality when the fish were challenged with *V. anguillarum*. In unvaccinated chum salmon challenged with *V. ordalii* the mortality rate was between 20 and 70%, and in those which were challenged with *V. anguillarum*, between 70 and 90%. Statistical analysis showed that the mortality rate was significantly higher in unvaccinated fish than in vaccinated fish in all treatment combinations. Within bacterial species, there was no significant difference in mortality rate caused by bacteria that were passed through English sole or chum salmon in both vaccinated and unvaccinated fish. The average mortality rates of both vaccinated and unvaccinated fish are given in Table 1.

In most treatments, mortality in unvaccinated fish began sooner (about two days after challenged) than in vaccinated fish (about three days after challenged). However, statistical analysis did not show that there was a significant difference in mean time to death between vaccinated and unvaccinated groups, or between *V. ordalii* and *V. anguillarum* within fish species. In English sole, mean time to death of vaccinated group was between 3.0 and 5.0 days, and in the unvaccinated group was between 2.3 and 7.3 days. In

unvaccinated chum salmon the mean time to death was between 5.4 and 8.5 days. The average mean time to death in each treatment is presented in Table 2.

Relative percent survival (RPS) of English sole challenged with *V. ordalii* ranged between 50 and 71.4% (average 65.2%), and with *V. anguillarum* was between 50 and 80% (average 69.7%).

Table 1.
Average mortality and relative percent survival (RPS) of vaccinated and unvaccinated English sole (*Parophrys vetulus*) and chum salmon (*Oncorhynchus keta*) challenged with *Vibrio ordalii* and *Vibrio anguillarum*.

Fish species/ Bacteria species	Average mortality rates (%) Vaccinated	Unvaccinated	RPS
English sole			
V.ordalii (E) ($5.5x10^7$)	15.0*)	45.0	66.7**)
(C) ($5.2x10^7$)	20.0	55.0	63.6
Average	17.5	50.0	65.2
V. anguillarum (E) ($4.8x10^7$)	15.0*)	55.0	72.7
(C) ($4.3x10^7$)	15.0	45.0	66.7
Average	15.0	50.0	69.7
Chum salmon			
V. ordalii (E)($5.0x10^6$)	0.0	30.0	100.0
(C)($5.0x10^6$)	0.0	60.0	100.0
Average	0.0	45.0	100.0
V. anguillarum (E) ($2.0x10^6$)	0.0	80.0	100.0
(C) ($2.6x10^6$)	5.0	80.0	93.8
Average	2.5	90.0	96.9

(E) : The bacteria were passed three times through English sole.
(C) : The bacteria were passed three times through Chum salmon
*) : There was significant difference in mortality rates between vaccinated and unvaccinated fish ($P<0.05$).
**) : There were two replicates per treatment. with 10 fish per replicate.

In chum salmon challenged with *V. o* the RPS was 100% (average 96. Chum salmon had a higher RPS thar English sole whether challenged witl *ordalii* or *V. anguillarum*. When comparation of RPS was made betw fish exposed to different RPS v observed. Exposure to bacteria that been passed through the heterologous species resulted in a lower RPS in cl salmon than did exposure to the bact that had been passed through fish spe (Table 1).

Both juvenile English sole and finge chum salmon produced antibody ti againts *V. ordalii* and *V. anguillarum* t terins, and titer level and durations w similar in both fish species (Figure 1 2). These fish species had higher antib titers (about twofold) when they w immunized with *V. anguillarum* bact than when *V. ordalii* bacterin was us Antibody titers were first detected 1 w after immunization, and reached a m mum in 3 or 4 weeks. After reaching pe 1:32 for *V. ordalii* bacterin, and1:64 for *anguillarum* bacterin), the antibody tit decreased slowly until the end of experiment. However, when experiment was terminated about 5 mon after immunization both English sole a chun salmon still had positive antib titers (between 1:2 and 1:4).

Some juveniles English sole (about 5-10 from both vaccinated and unvaccinat groups died about one month af immunization. Renal tissue was innocul ed onto TSA and no bacterial infectic were detected. The dead fish were usul thin and the digestive tract contained food suggesting that they were not feedi and starved to death.

Table 2.
Average mean time to death of vaccinated and unvaccinated English sole (*Parophrys vetulus*) and chum salmon (Oncorhynchus kate) challenged with *Vibrio ordalii* and *Vibrio anguillarum*.

Fish species/ Bacteria species	Average mean time to death (days)	
	Vaccinated	unvaccinated*)
English sole		
V. ordalii (E) ($5.5x10^7$)	3.5	2.6**)
(C) ($5.2x10^7$)	4.0	3.1
Average	3.75	2.95
V. anguillarum (E) ($4.8x10^7$)	3.5	4.1
(C) ($4.3x10^7$)	3.5	5.8
Average	3.50	4.95
Chum salmon		
V. ordalii (E) ($5.0x10^6$)	-***)	7.3
(C) ($5.0x10^6$)	-	5.4
Average	-	6.35
V. anguillarum (E) ($2.0x10^6$)	-	5.7
(C) ($2.6x10^6$)	-	6.5
Average	3.0	6.1

(E) : The bacteria were passes three times through English sole
(C) : The bacteria were passes three times through Chun salmon
*) : There was significant difference in mean time to death between vaccinated and unvaccinated fish ($P<0.05$).
**) : There were two replicates per treatment, with 10 fish per replicate.
***) : There was no fish mortality.

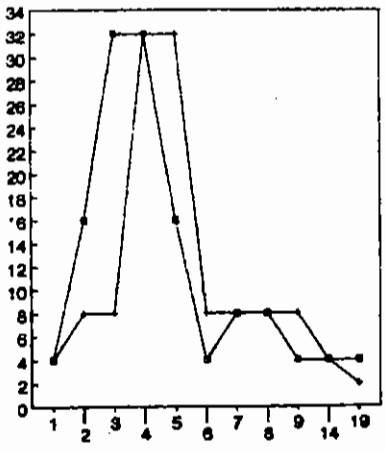

Figure 1.
Antibody titers of chum salmon (*Oncorhynchus keta*) and English sole (*Parophrys vetulus*) serum after immunization with *Vibrio ordalii* bacterin.

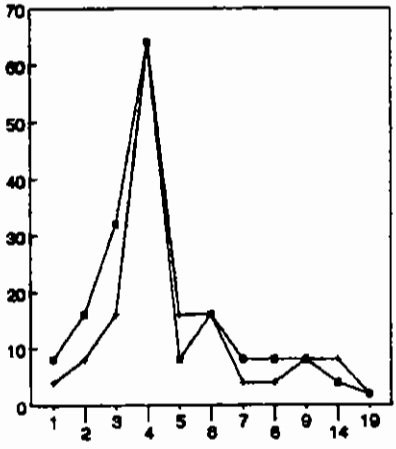

Figure 2.
Antibody titers of chum salmon (*Oncorhynchus keta*) and English sole (*Parophrys vetulus*) serum after immunization with *Vibrio anguillarum* bacterin.

Discussion

Different fish species may develop different levels of protection even though they were immunized with the same bacterin and have the same optimum temperature ranges. In oral immunization studies using four species of marine tropical fish, Prescott (1977) found that the protection against *V. anguillarum* for each of the four species tested varied to considerable degree. Other investigators have done similar experiments and found the results to vary from one experiment to another. The results were affected by environmental conditions especially water temperature, fish species, size of fish, type *of V. anguillarum* bacterins and immunization methods (Groberg, 1981; Tatner and Horne, 1983; Estavez, *et al.*, 1994).

In the experiment conducted to determine the duration of antibody titers and the immune response of English sole and chum salmon after immunization by intraperitoneal injection, differences in protection were observed. In general, immunization gave protection to both Englis sole and chum salmon and mortality rates of immunized fish were significantly lower than controls.

However, when a comparison was made between the two fish species, English sole had a lower relative percent survival (RPS) than chum salmon. This indicated that English sole had a lower level of protective immunity than chum salmon even though they had similar antibody titers. Antibody titers had a different meaning in different fish species, and do not necessarily serve as a direct indication of protection level.

Similar results were found by Spence *et al.*, (1965) in studies of rainbow trout immunized *against Aeromonas salmonicida.* They suggested that although agglutinins may be present in sera at high levels, this does not necessarily indicated the existence of protecting antibodies. In contrast, Laurencin and Tangtrongpiros (1980). Aoki *et al.* (1984) could not detect agglutinating antibodies in fish sera which were immunized by oral or direct immersion with *V. anguillarum* bacterins. However they did find significant differences in mortality rates between immunized fish and control after being challenged by water-borne exposure or intraperitoneal injection with *V. anguillarum* with mortality rates of immunized fish generally lower than controls.

Although the immunized English sole and chum salmon had significantly lower mortality rates than did the controls, they did not have a significantly longer mean time to death. It is posisble that in this experiment the immune response only helps to prevent infection and if the fish become infected, the development of the disease is the same in both immunized and control fish. In a similar study with *A. salmonicida*, Spence *et al.* (1965) found that immunization not only decreased mortality rates, but also delayed the mean time to death as compared to the controls.

The peaks of antibody titers observed in this experiment were subtantially lower than the peaks of antibody titers found by most other investigators. One of the reasons may be the size of fish used in the experiments which was much smaller than the average sizes fish used by other investigators and could influence the immune response (Johnson *et al.*, 1982b; Tatner and Horne, 1984). When the experiment was terminated 19 weeks after immuniztion, both English sole and chum salmon still had positive antibody titers (about 1:2 to 1:4). However, the fish were not challenged and the level of protection against vibriosis is not known.

Acknowledgment

I would like to thank the Rockefeller Foundation for the financial support which they provided and Dr. Robert E. Olson for excellent guidance in this research.

Literatures Citted

Amend, D. F., and K.A. Johnson, 1984. Evidence for lack antigenic competition among various combinations of *Vibrio anguillarum. Yersinia ruckeri. Aeromonas salmonicida* and *Renibacterium salmoninarum* bacterins when administrated to salmonid fishes. J. Fish. Dis., 7 : 293-299.

Aoki, T., T. Kitao, M. Fukuda, S. Takahashi, and Segusa,1984. Modification of the hyperosmotic infiltration method of vaccination against vibriosis in cultured ayu, *Plecoglossus altivelis* Temmic and Schlegel. J. Fish Dis., 7: 149-156.

Egidius and Anderson, 1984. Disease problem in cod rearing. Inst. Mar. Res., ed. by E.Dahl, D.S. Danielssen, E. Moksness, and P. Solemdal, Flodevigen Biol. Sta., Arendal, 761-769.

Estavez, J., J. Leiro, A.E. Toranzo, J.L. Barja, and F.M. Ubeira, 1994. Role of Serum anti bodies in protection of vaccinated turbot *(Scophthalmus maximus)* against vibriosis. Aquaculture 123: 197-204.

Gould, R.W., 1977. Development of a new vaccine delivery system for immunizing fish and investigation of the protective antigens in *Vibrio anguillarum.* Ph.D. thesis, Oregon State University, Corvallis, 145 p.

Groberg, W.J., Jr., 1981. Infection and the immune response induced by *Vibrio anguillarum* in juvenile coho salmon (*Oncorhynchus kisutch*). Ph.D. thesis, Oregon State University, Corvallis, 107 p.

Grondel, J.L. and H.J.A.M. Boesten, 1982. The influence of antibiotics on the immune system I. Inhibition of the mitogenic leukocyte response *in vitro* by oxytetracycline. Dev. Comp. Immunol., Sup. 2,211-216.

Hahnel, G.B. and R.W. Gould, 1982. Effects of temperature on biochemical reactions and drug resistance of virulent and avirulent *Aeromonas salmonicida.* J. Fish Dis., 5:329-337.

Itami, T. and R. Kusuda, 1980. Studies on spray vaccination against vibriosis in cultured ayu. II: Duration of vaccination efficacy and effect of different vaccine preparation. Bull. Jap. Soc. Sci. Fish 46 (8): 699-703.

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 Dis., 5: 197-205.

Johnson, K.A.; J.K. Flynn, and D.F. Amend, 1982b. Duration of immunity in salmonid vaccinated by direct immersion with *Yersinia ruckeri* and *Vibrio anguillarum* bacterins. J. Fish Dis., 5: 207-213.

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. Jap. Soc. Sci. Fish., 50 (5): 771-774.

Kerry, J., M. Hiney, R. Coyne, D. Cazabom, S. Nic Gabhainn and P. Smith, 1994. Frequency and distribution of resistance to oxytetracycline in microorganisms isolated from marine fish farm sediments following therapeutic use of oxytetracycline. Aquaculture 123:43-54.

Laurencin, F.B. and J. Tangtrongpiros, 1980. Some results of hyperosmotic infiltration in Brittany. 60-68, in: Fish diseases, W. Ahne (ed.) third COP-RAQ-session, Springer-Verlag, Berlin.

Lewis, E.H.; R.J. Tarpley, J.T. Marks and R.F. Sis, 1985. Drug induced structural changes in olfactory organs of channel catfish *Ictalurus punctatus*. J. Fish. Biol., 26: 355- 358.

Limsuwan, C., 1993. Diseases of black tiger shrimp (*Penaeus monodon Febricus)* in Thailand. Tech. Bull., American Soybean Assoc. 1-22.

Lupiani, B., A.M. Baya, B. Magarinos, J.L. Romalde, T. Li, B.S. Roberson, F.M. Hetrick and A.E. Toranso, 1993. *Vibrio mimicus* and *Vibrio cholerae* non-01 isolated from wild and hatchery reared fish. Fish Pathol. 28 (1): 15-26.

Nash, G., C. Nithimathachoke, C. Tungmandi, A. Arkarjamorn, P. Pratanpitat, and P. Ruamthaveesub, 1990. Vibriosis and its control in pond-reared *Penaeus monodon* in Thailand. Diseases in Asian Aquaculture I.M. Shariff, R.P. Subasinghe, J.R. Arthur (eds): 143-155.

Prescott, S.R., 1977. A technical report on oral immunization of marine tropical fish *against Vibrio anguillarum*. Aquaculture, 11: 281-283.

Richards, R.H., 1980. Observations on vibriosis in cultured flatfish. 75-81, in: Fish diseases, W. Ahne (ed.), third COMPRAQ-Session, Springer-Verlag, Berlin.

Rohovec, J.S., J.R. Winton and J.L. Fryer, 1981. Bacterins and vaccines for control of infectious diseases in fish. In: Poc. Rep. of China-United States Cooperative Science Seminar on Fish Diseases. G. H. Kou, J.L. Fryer, and M.L. Landolt (eds.), Net. Sci Council Taipei, 115-121.

Sakai, M. T. Aoki, T. Kitao, J.S. Rohovec and J.L. Fryer, 1984. Comparisons of the cellular immune response of fish vaccinated by immersion and injection *of Vibrio anguillarum*. Bull. Jap. Soc. Fish., 50 (7): 1187-1192.

Spence, K.D.; J.L. Fryer, and K.S. Pilcher, 1965. Passive immunization of certain salmonid fishes against *Aerominas salmonicida*. Can. J. Microbiol., 11 (3): 397- 405.

Tabata, K.S. S. Karat and M.S. Ruiz, 1982. Studies on naturally accurring disease during the production of ayu (*Plecoglossus altiveli*) in seawater. II: Dynamics of *Vibrio anguillarum*. Fish Pathol., 17 (3): 205-212.

Tatner, M.F. and M.T. Horne, 1983. Factors influencing the uptake of 14C-labelled *Vibrio anguillarum* vaccine in direct immersion experiments with rainbow trout, *Salmon gairdneri*. J. Fish. Biol., 22:585-591.

Tatner, M.F. and M.T. Horne, 1984. The effects of early exposure to *Vibrio anguillarum* vaccine on the immune response of the fry of the rainbow trout, *Salmo gairdneri*., Fish Biol. 41: 193-202.

Viele, D. T.H. Kerstetter and J. Sullivan, 1980. Adaptive transfer of immunity against *Vibrio anguillarum* in rainbow trout *Salmo gairdneri*., vaccinated by the immersion method. J. Fish Biol., 17: 379-386.