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The Non-specific Immunostimulation and Adjuvant Effects of *Vibrio anguillarum* Bacterin, M-glucan, Chitin and Freund's Complete Adjuvant against *Pasteurella piscicida* Infection in Yellowtail

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The non-specific immunostimulation and adjuvant effects of *Vibrio anguillarum* bacterin (VAB), M-glucan, chitin and Freund's complete adjuvant (FCA) against pasteurellosis were examined in yellowtail, *Seriola quinqueradiata*. Control fish were injected intraperitoneally with 0.1 m*l* of sterile phosphate buffered saline (PBS). Experimental fish were injected similarly with either vaccine (lipopolysaccharide-mixed chloroform-killed *Pasteurella piscicida*, LPS-CKC), an immunostimulant or mixture of the vaccine and an immunostimulant. All fish were subsequently challenged by the same but live *P. piscicida* strain using an immersion method at 15, 25, 35 and 45 days post-inoculation. Immunostimulants alone were found to slightly increase the protection against Pasteurella infection, but the effects were not statistically significant. The most effective adjuvant was FCA, however the enhanced protection was not statistically different from that of fish inoculated with LPS-CKC alone. However, the relative percent survival (RPS) of fish vaccinated with M-glucan, chitin or Vibrio bacterin as adjuvant was lower than that of fish vaccinated with only LPS-CKC bacterin alone. Thus, M-glucan, chitin and *Vibrio* bacterin had no positive adjuvant effects on the *P. piscicida* vaccine.

Key words: vaccine, immunostimulant, yellowtail, *Pasteurella piscicida bacterin*, M-glucan, chitin, Freund's complete adjuvant

Pasteurellosis, caused by *Pasteurella piscicida*, is one of the major bacterial diseases responsible for serious economic losses in many species of wild and farmed fish in Asia, USA and Europe, and is a very important disease in yellowtail *Seriola quinqueradiata* culture in Japan (Kitao, 1993). The pathogen was identified as *P. piscicida* (Kusuda and Yamaoka, 1972), but recently Gauthier *et al.* (1995) proposed the name *Photobacterium damsela* subsp. *piscicida* for this pathogen based on an analysis of ribosomal 16S RNA sequences.

Several vaccines have been tested for the control of pasteurellosis, including whole-cell bacterins (Fukuda and Kusuda, 1981), attenuated live vaccines (Kusuda and Hamaguchi, 1988), lipopolysaccharide (LPS) extracts (Fukuda and Kusuda, 1982; Fukuda and Kusuda, 1985), ribosomal vaccine (Kusuda *et al.*, 1988) and toxoid-enriched whole-cell vaccine (ECP) (Magarinos *et al.*, 1994). Previously, we developed an LPS mixed chloroform-killed bacterin (LPS-CKC) against *P. piscicida* and this vaccine preparation elicited an excellent immune response in yellowtail (Kawakami *et al.*, 1997). In addition, this vaccine was effective against experimental and natural infections (Kawakami *et al.*, 1997).

Immunostimulants may increase the organism's resistance against disease by enhancing the non-specific immune system. In fish, several immunostimulants such as Freund's complete adjuvant (FCA) (Olivier *et al.*, 1985; Kajita *et al.*, 1992), FK-565 (Kitao and Yoshida, 1986), levamisole (Siwicki, 1987; Kajita *et al.*, 1990), β-1,3-glucans (Yano *et al.*, 1989; Robertsen *et al.*, 1990) and chitin (Sakai *et al.*, 1992) have been reported to contribute to the control of diseases in fish culture. Immunostimulants may increase not only non-specific immunity but also specific immune responses in fish (Anderson, 1992). The present study investigated whether inoculation of the immunostimulants enhances the profective efficacy of LPS-CKC vaccine against experimental *P. piscicida* infection in yellowtail.

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Materials and Methods

Fish

Yellowtail were obtained from a fish farm in Uwajima Bay, Ehime, Japan. No occurrence of pasteurellosis has been observed in the farm from where the fish were obtained. A total of 3,000 fish with a mean weight of 8 g were used in this study. Examination of 10 fish for the presence of *P. piscicida* using tryptone soya agar (TSA) (Nissui) supplemented with 2.0% NaCl (2% NaCl-TSA) revealed no bacteria in their kidneys. The fish were maintained in fiberglass containers supplied with sea water for 2 wk at 24.0°C and fed daily with commercial pellets.

Immunostimulants

Vibrio anguillarum bacterin (VAB) (Kyouritsusyoji, Japan), M-glucan (Nissin-fine chemicals, Japan), chitin (Mitaka Seiyaku, Japan) and FCA (Difco, USA) were used as immunostimulants. M-glucan, chitin and VAB were suspended in physiological saline and adjusted to 640 μg/m*l*, 4 mg/m*l* and 10^6 cfu/m*l*, respectively.

Bacterin

The *P. piscicida* strain PS91-142 isolated from diseased yellowtail was used in this study. This strain was cultured on 2.0% NaCl-brain heart infusion (Nissui) (NaCl-BHI) agar for 2 days at 25°C and collected using an inoculating loop. The LPS-mixed chloroform-killed (LPS-CKC) bacterin was prepared by the method of Kawakami *et al.* (1997).

Administration of immunostimulants and vaccine

An individual immunostimulant (0.1 m*l*) or FCA (0.1 m*l*) was intraperitoneally injected to 300 fish. The same volumes of the LPS-CKC bacterin and each immunostimulant were mixed. Then, yellowtail were injected intraperitoneally with 0.1 m*l* of each LPS-CKC bacterin-immunostimuant mixture. Control fish were injected with 0.1 m*l* of LPS-CKC bacterin alone or sterile phosphate-buffered saline (PBS). One hundred fifty fish were used in each group. Control and experimental fish were maintained in separate fiberglass containers supplied with sea water at 23.0 ± 2.5°C until experimental challenge.

Experimental challenge

Fifteen, 25, 35 and 45 days after vaccination, 25 yellowtail from each experimental group were challenged by immersion into seawater containing live *P. piscicida* SP91-142 for 5 min at 22°C. The challenge doses used were 10^2 or 10^3 cfu/m*l*. Fish were kept at 22°C and observed for up to 10 days. Fish that died were necropsied and kidney tissue materials were inoculated onto 2% NaCl-TS agar to determine the presence of *P. piscicida*.

Statistics

The efficacies of the vaccine and the immunostimulants were determined by calculating the relative percent survival (RPS) according to Amend (1981) and significantly determined by the chi-square test. The chi-square test was also used to evaluate the efficacy of the adjuvant effects in vaccinated groups.

Results

The RPSs of fish groups challenged by *P. piscicida* at the dose of 10^2 cfu/m*l* are shown in Fig. 1. The efficacy of adjuvant free vaccine continued for 35 days after vaccination. The supplemental adjuvant effect was observed only in the group injected with FCA, however the enhanced protection by FCA was not significantly higher than that immunized with LPS-CKC alone. The RPSs of fish groups injected with M-glucan, chitin or *Vibrio* bacterin as adjuvants were lower than that of fish group vaccinated with LPS-CKC bacterin alone. When fish were injected with immunostimulants alone (VAB, M-glucan, chitin and FCA), they slightly increased the protection against *Pasteurella* infection, but their effects were not statistically significant.

The RPSs of fish groups challenged at the dose of 10^3 cfu/m*l* are shown in Fig. 2. The efficacy of adjuvant-free vaccine also observed until 45 days after vaccination. The supplemental efficacy of the adjuvant was shown only in the FCA-treated fish groups, however, the enhanced protection was not significant by different compared with vaccine alone.

P. piscicida was isolated from the kidneys of all dead fish in these artificial challenge experiments.

Discussion

Sakai *et al.* (1995) and Adams *et al.* (1988) reported that rainbow trout *Oncorhynchus mykiss* immunized with VAB showed the increased protection against *Streptococcus* sp. challenge and furunculosis, respectively. *V. anguillarum* LPS can stimulate the phagocytic activity and the production of superoxide anion in Atlantic salmon macrophages *in vitro* (Dalmo and

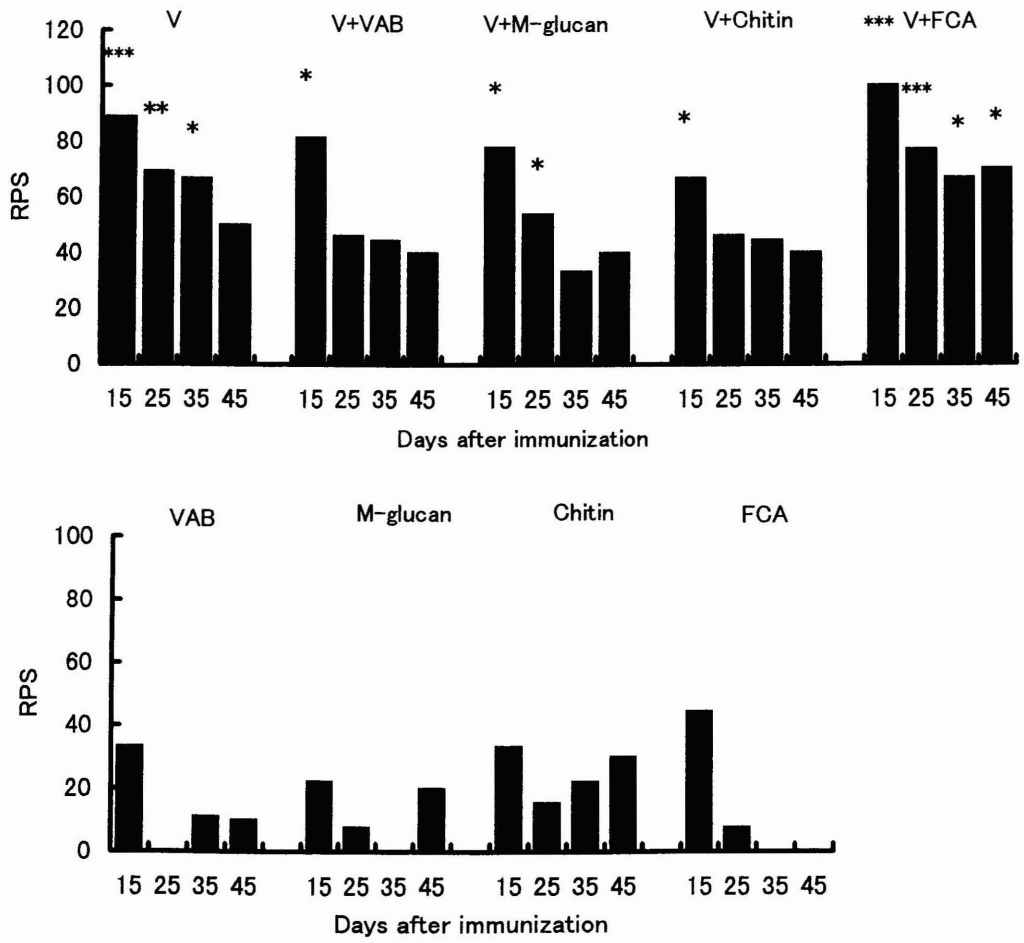

Fig. 1. The RPSs (relative percent survival) of fish groups (N = 25) challenged by *Pasteurella piscicida* at the dose of 10^2 cfu/m*l*. Asterisk indicates significant difference from control groups: * ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.005$). V: Vaccine (LPS-CKC), VAB: *Vibrio anguillarum* bacterin, FCA: Freund's complete adjuvant.

Seljelid, 1995). We observed slight, but not significant, stimulation of non-specific protection in the VAB-injected fish against *P. piscicida* infection, but, no adjuvant effects in fish vaccinated with the LPS-FKC and VAB cocktail. The same phenomenon has been reported for fish vaccinated with an *A. salmonicida* whole cells and VAB cocktail (Adams *et al.*, 1988).

M-glucan stimulates the specific and non-specific immune responses of fish (Robertsen *et al.*, 1994). Fish injected with glucan were found to have an increased protection against *V. anguillarum*, *V. salmonicida*, *Yersinia ruckeri* (Robertsen *et al.*, 1990) or *Edwardsiella ictaluri* (Chen and Ainsworth, 1992). In the present study, injection of M-glucan decreased, though not significantly, the mortality of yellowtail against artificial *P. piscicida* challenges, with the exception of one group (challenged with 10^2 cfu/m*l* 35 days after injection). Matsuyama *et al.*, (1992) reported that injection of schizophyllan and scleroglucan, another type of glucan, to yellowtail did not provide protection against *P. piscicida*. In our study, M-glucan was not found to enhance the efficacy of LPS-CKC vaccine.

Chitin is a polysaccharide forming the principal component of crustacean shells, insect exoskeletons and the cell walls of certain fungi. Sakai *et al.* (1992) reported that the injection of chitin in rainbow trout stimulated macrophage activities and increased the resistance to *V. anguillarum* infection. The yellowtail injected with

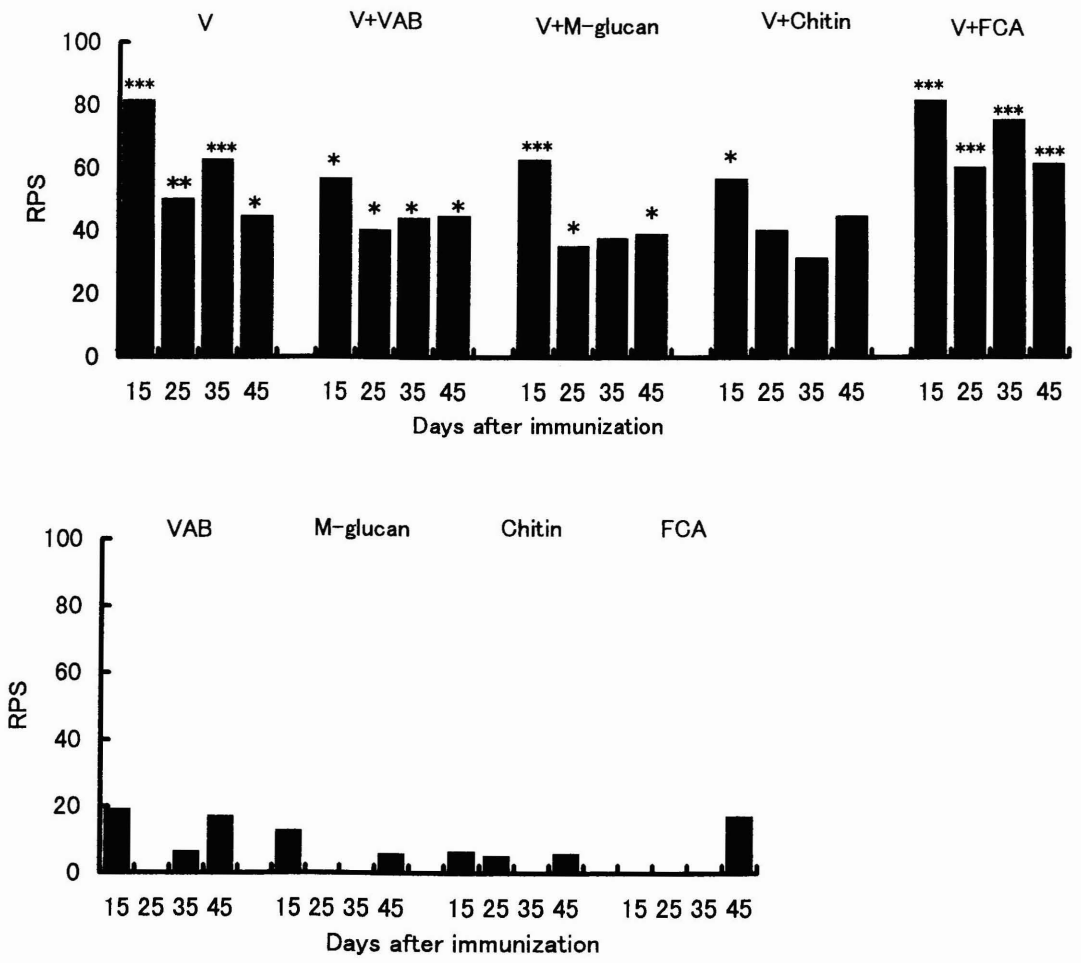

Fig. 2. The RPSs of fish groups (N = 25) challenged by *Pasteurella piscicida* at the dose of 10^2 cfu/ml. Asterisk indicates significant difference from control groups: * ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.005$). V: Vaccine (LPS-CKC), VAB: Vibrio anguillarum bacterin, FCA: Freund's complete adjuvant.

chitin alone in the present study also appeared to have an increased protection to *P. piscicida* challenge and this continued for at least 45 days after treatment, however the difference from control fish groups was not statistically significant. In spite of the increase of non-specific protective immunity in yellowtail injected with chitin, no adjuvant effects of chitin were observed. The adjuvant effect of chitin has not been reported in fish, but has been observed in mice and guinea pigs (Nishimura *et al.*, 1985).

FCA which is composed of a mixture of killed *Mycobacterium tuberculosis* and mineral oil, can be used to emulsify the antigen, and the resulting enhancement of the immune response is well known in mammals. The injection of FCA alone in fish can also stimulate non-specific immunity and increase the resistance to fish pathogens (Olivier *et al.*, 1985; Adams *et al.*, 1988; Kajita *et al.*, 1992). Olivier *et al.* (1985) reported that coho salmon, *O. kisutch*, injected with FCA showed high levels of protection against *A. salmonicida* (LD_{50} increase of 450-fold) and *V. ordalii* (LD_{50} increase of 560-fold) and a low level of protection against *A. hydrophila* (LD_{50} increase of only 5.3-fold).

In this study, yellowtail injected with LPS-CKC bacterin in FCA emulsion showed an enhanced higher protection against *P. piscicida* infection, compared to those inoculated with LPS-CKC bacterin alone. Fukuda and Kusuda (1981) reported that yellowtail injected with

formalin-killed *P. piscicida* bacterin-emulsifying FCA showed a greater protection to artificial challenges and that the agglutinating antibody leavels in serum were also elevated.

Our previous study (Kawakami *et al.*, 1997) showed that the efficacy of the LPS-CKC vaccine continued for at least 35 days after immunization. In the current study, the efficacy was somewhat reduced at 45 days after vaccination in fish vaccinated by bacterin alone (RPSs were 50.0 and 44.4). However, vaccine efficacy was still observed in fish 45 days after vaccination when inoculated with bacterin-emulsifying FCA (RPSs were 70.0 and 61.1). This prolonged efficacy of the vaccine should be useful for yellowtail culture.

Acknowledgments

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References

Adams, A., N. Auchinachie, A. Bundy, M. F. Tatner and M. T. Horne (1988): The potency of adjuvanted injected vaccines in rainbow trout (*Salmo gairdneri* Richardson) and bath vaccines in Atlantic salmon (*Salmo salar* L.) against furunculosis. *Aquaculture*, **69**, 15–26.

Amend, D. F. (1981): Potency testing of fish vaccines. In "Biological standarization, Fish biologics: Serodiagnostics and Vaccines". *Dev. Biol. Standard.*, **49**, S. Karger, New York, pp. 447–454.

Anderson, D. P. (1992): Immunostimulants, adjuvants, and vaccine carriers in fish: applications to aquaculture. *Ann. Rev. Fish Dis.*, **2**, 281–307.

Chen, D. and A. J. Ainsworth (1992): Glucan administration potentiates immune defence mechanisms of channel catfish, *Ictalurus punctatus* Rafinesque. *J. Fish Dis.*, **15**, 295–304.

Dalmo, R. A. and R. Seljelid (1995): The immunomodulatory effect of LPS, laminaran and sulfated laminaran [β(1,3)-D-glucan] on Atlantic salmon, *Salmo salar* L., macrophages *in vitro*. *J. Fish Dis.*, **18**, 175–185.

Fukuda, Y. and R. Kusuda (1981): Efficacy of vaccination against pseudotuberculosis in cultured yellowtail by various routes of administration. *Bull. Japan. Soc. Sci. Fish.*, **47**, 147–150.

Fukuda, Y. and R. Kusuda (1982): Detection and characterization of precipitating antibody in the serum of immature yellowtail immunized with *Pasteurella piscicida* cells. *Fish Pathol.*, **17**, 125–127.

Fukuda, Y. and R. Kusuda (1985): Vaccination of yellowtail against pseudotuberculosis. *Fish Pathol.*, **15**, 421–425.

Gauthier, G., B. Lafay, R. Ruimy, V. Brettmayer, L. J. Nicolas, M. Gauthier and R. Christen (1995): Small-subunit rRNA sequences and whole DNA relatedness concur for the reassignment of *Pasteurella piscicida* (Snieszko *et al.*) Janssen and Surgalla to the genus *Photobacterium* as *Photobacterium damsela* subsp. *piscicida* comb. nov. *Int. J. Syst. Bacteriol.*, **45**, 139–144.

Kajita, Y., M. Sakai, S. Atsuta and M. Kobayashi (1990): The immunomodulatory effects of levamisole on rainbow trout, *Oncorhynchus mykiss*. *Fish Pathol.*, **25**, 93–98.

Kajita, Y., M. Sakai, S. Atsuta and M. Kobayashi (1992): Immunopotentiation activity of Freund's complete adjuvant in rainbow trout *Oncorhynchus mykiss*. *Nippon Suisan Gakkaishi*, **58**, 433–437.

Kawakami, H., N. Shinohara, Y. Fukuda, H. Yamashita, H. Kihara and M. Sakai (1997): The efficacy of lipopolysaccharide mixed chloroform-killed cell (LPS-CKC) bacterin of *Pasteurella piscicida* on Yellowtail, *Seriola quinqueradiata*. *Aquaculture* **154**, 95–105.

Kitao, T. (1993): Pasteurellosis, In "Bacterial diseases of fish" (ed. by V. Inglis, R. J. Roberts and N. R. Bromage), Blackwell, Oxford, pp. 159–165.

Kitao, T. and T. Yoshida (1986): Effect of an immunopotentiator on *Aeromonas salmonicida* infection in rainbow trout (*Salmo gairdneri*). *Vet. Immunol. Immunopathol.*, **12**, 287–291.

Kusuda, R. and M. Yamaoka (1972): Etiological studies on bacterial pseudotuberculosis in cultured yellowtail with *Pasteurella piscicida* as causative agent. I. On the morphology and biochemical properties. *Bull. Japan. Soc. Sci. Fish.*, **38**, 79–83.

Kusuda, R. and M. Hamaguchi (1988): The efficacy of attenuated live bacterin of *Pasteurella piscicida* against pseudotuberculosis in yellowtail. *Bull. Eur. Ass. Fish Pathol.*, **8**, 50–52.

Kusuda, R., M. Ninomiya, M. Hamaguchi and A. Muraoka (1988): The efficacy of ribosomal vaccine prepared from *Pasteurella piscicida* against pseudotuberculosis in cultured yellowtail. *Fish Pathol.*, **23**, 191–196.

Magarinos, B., J. L. Romalde, Y. Santos, J. F. Casal, J. L. Barja and A. E. Toranzo (1994): Vaccination trials on gilthead seabream (*Sparus aurata*) against Pasteurella piscicida. *Aquaculture*, **120**, 201–208.

Matsuyama, H., R. E. P. Mangindaan and T. Yano (1992): Protective effect of schizophyllan and scleroglucan against *Streptococcus* sp. infection in yellowtail (*Seriola quinqueradiata*). Aquaculture, **101**, 197–203.

Nishimura, K., S. Nishimura, N. Nishi, F. Numata, Y. Tane, S. Tokura and I. Azuma (1985): Adjuvant activity of chitin derivatives in mice and guinea-pigs. *Vaccine*, **3**, 379–383.

Olivier, G., T. P. T. Evelyn and R. Lallier (1985): Immunity to *Aeromonas salmonicida* in coho salmon (*Oncorhynchus kisutch*) induced by modified Freund's complete adjuvant: its non-specific nature and the probable role of macrophages in the phenomenon. *Dev. Com. Immunol.*, **9**, 419–432.

Røbertsen, B., G. Rørstad, R. Engstad and J. Raa (1990): Enhancement of non-specific disease resistance in Atlantic salmon, *Salmo salar* L., by a glucan from Saccharomyces cerevisiae cell walls. *J. Fish Dis.*, **13**, 391–400.

Røbertsen, B., R. E. Engstad and J. B. Jørgensen (1994): β-glucan as immunostimulants in fish. *Modulators of Fish*

Immune Responses, **1**, 83–99.

Sakai, M., H. Kamiya, S. Ishii, S. Atsuta and M. Kobayashi (1992): The immunostimulating effects of chitin in rainbow trout, *Oncorhynchus mykiss*. In "Diseases in Asian aquaculture I" (ed. by M. Shariff, R. P. Subasighe and J. R. Arthur), Fish Health Section, Asian Fish. Soc., Manila, pp. 413–417.

Sakai, M., S. Atsuta and M. Kobayashi (1995): Efficacies of combined vaccine for *Vibrio anguillarum* and *Streptococcus* sp. *Fish. Sci.*, **61**, 359–360.

Siwicki, A. (1987): Immunomodulating activity of levamisole in spawners of carp *(Cyprinus carpio* L.). *J. Fish Biol.*, **31**, 245–246.

Yano, T., R. E. P. Mangindaan and H. Matsuyama (1989): Enhancement of the resistance of carp *Cyprinus carpio* to experimental *Edwardsiella tarda* infection, by some β-1,3-glucans. *Nippon Suisan Gakkaishi*, **55**, 1815–1819.