Mar. Res. Indonesia Vol.34, No.2, 2009: 81-85

THE EFFECT OF VITAMIN C AND *AEROMONAS* VACCINE ON THE IMMUNE RESPONSE AND DISEASE RESISTANCE OF GROUPER (*EPINEPHELUS FUSCOGUTTATUS*)

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Received: 24 October 2008 Revised and Accepted: 4 September 2009

ABSTRACT

We evaluated the effectiveness of vitamin C and *Aeromonas salmonicida* vaccine in grouper (*Epinephelus fuscoguttatus*) for increasing immune responses and protection against *A.salmonicida*. The vitamin C used was polyethoxylated ascorbic and tocopherol. The vaccine was prepared from formalin-killed cells and concentrated extracellular products of a single isolate *A.salmonicida*. Bath immersion vitamin C and vaccine trials were conducted for 60 min. Fish used had a mean weight 25 g. Control groupers were injected with tryptic soy broth. The results showed that vitamin C enhanced phagocytic activity in head kidney leucocytes of grouper 7, 14, 28 and 36 days after treatments. A significant different of the antibody titre was found between control fish and the treated fish at 42 days after treatments. In addition, at day 42, Relative Percent Survival (RPS) for control group was 53.3 %, vitamin C-treated group was 80.0 % and vaccinated group was 90.0 %. The results of this study suggest that bath immersion of vitamin C provided an increasing of phagocytic activity (non-specific immune responses), titre antibody (specific immune responses) and protection against *A. salmonicida* infection in grouper. *A. salmonicida* vaccine also enhanced titre antibody and protection against *A. salmonicida* infection in grouper.

Keywords: Immunostimulant, Vitamin C, vaccine, grouper.

INTRODUCTION

The outbreak of diseases is a limiting factor in fish culture. The high susceptibility of fish to stress and the rapid spread of diseases in water have forced aquaculturists to concentrate their efforts on maintaining their fish in good health in order to achieve sustainable economic performances. Growing healthy fish requires them to be able to develop strong defence mechanisms against pathogen invasion. These are the non-specific and the specific immune response. The non-specific immune response is more important in fish than it is in mammals. Improving the immune response leads to a better vaccination efficiency. Vaccines induce a specific immune response and an increased capacity to kill the pathogens by non-specific defence mechanisms.

At many farms and hatcheries several antibiotics, vaccines, and chemotherapeutic agents as well as some immunostimulants have been used to prevent viral, bacterial, parasitic and fungal diseases. Whilst vaccination is the method of choice over antibiotic treatments for the control of many fish diseases, vaccines for others are unavailable or, at best, in the early stages of their development. In recent years in the aquaculture industry, increasing consideration has been given to the use of immunostimulants as adjuncts to vaccination and as a potential route to the reduction in the widespread use of antibiotics.

Vitamin C is an essential vitamin for normal growth and physiological function of fish. It functions as a general water-soluble redox reagent, on collagen formation (Sato *et al.*, 1982) iron metabolism and hematology (Sandnes *et al.*, 1990) and

DOI: 10.14203/mri.v34i2.512

stress (Wedemeyer, 1969). Its lack in the diet depressed growth rate, immunocompetence and increased susceptibility to bacterial diseases. Furthermore, immune-stimulating effects and increased resistance in fish fed elevated levels of vitamin C have been demonstrated in channel catfish (Durve and Lovell, 1982) and Atlantic salmon (Hardie *et al.*, 1991). Grouper (*Epinephelus fuscoguttatus*) is one of the most economically important cultured marine fish in Asia, particularly Thailand, Malaysia, Philippines, Singapore, Indonesia and Taiwan. Because of their desirable taste, hardiness in a crowded environment and rapid growth, they are a good candidate for intensive aquaculture. However, information of vitamin C as an immunomodulator and vaccines based on killed pathogens in grouper is scarce. In Indonesia, the causative agents of diseases have been identified in detail. *Aeromonas salmonicida* is one of the main species of bacteria isolated from infected fish. Vaccine against *Aeromonas salmonicida* infection has not yet been developed.

The combination of good management, vaccination and immunostimulant treatments will insure higher survival rates in farming systems. The purpose of this study was to estimate the effects of vitamin C and *Aeromonas salmonicida* vaccine on the immune responses and disease resistance in grouper (*Epinephelus fuscoguttatus).*

MATERIALS AND METHODS

Animals and Rearing Conditions

Grouper, *Epinephelus* sp (mean weight 25 gr) were obtained from Takalar Fisheries Station, Takalar, South Sulawesi, Indonesia and kept in 80 l fiberglass tanks.

Vitamin C and *Aeromonas salmonicida* Vaccine

Vitamin C used for experiments were polyethoxylated ascorbic and tocopherol. These vitamins were purchased from PT. Sanbe Varma, Indonesia.

For the vaccine preparation, *Aeromonas salmonicida* was isolated from infected grouper and was grown in nutrient broth (NB) and incubated in a shaker (150 RPM) water bath at 27 °C for 72 hours. Cultures were treated with a final 3 % neutral buffered formalin concentration for 24 h. The formalin-treated culture was centrifuged at 7000 × g for 30 min, and cell pellet and culture fluid separated. Sixteen ml of the formalin-killed cells were added to 11 of the sterilized concentrated cell-free culture fluid. The vaccine had an optical density of 1.9 at 540 nm. The number of colony-forming units (CFU) ml^{-1} of *A.salmonicida* in the final vaccine preparation were estimated to be 4×10^9. The bacterial concentration was estimated by taking the optical density of the vaccine prior to killing in formalin. The actual number of CFU ml^{-1} was determined using a spiral autoplater and Qcount. The vaccine was determined to be sterile by lack of bacterial growth on sheep blood agar after 72 h incubation.

Bath Immersion Administration

Sixty grouper were divided into three groups of 20 fish each. Control group (Group A) were immersed in 11 of 500 ml sterile water: 500 ml tryptic soy broth (TSB) for 60 min, following immersion, the fish were placed in three replicate aquaria. Group B were immersed in 1000 mg vitamin C kg^{-1} of feed for 60 min, following immersion, the fish were placed in three replicate aquaria. Immunized fish (Group C) were immersed in undiluted vaccine containing 16 ml bacterin and 1000 ml toxoid for 60 min and, following immersion, the fish were placed in three replicate aquaria. Each group was sampled at 7, 14, 21, 28, 36 and 42 days after treatments.

Isolation of Head Kidney Cells

Grouper head kidney cells was removed and pushed through a nylon mesh with RPMI 1640 medium (Nissui, Japan) containing 1 % streptomycin/penicillin (S/P, Gibco, USA) and 0.2 % heparin (Sigma, USA). The cell suspension was then centrifuged at 500 × g for 5 min and washed three times with the same medium. Viable phagocytic cells, including neutrophils and macrophages, were counted by Trypan Blue Exclusion.

Blood and Serum Samples

Blood samples were collected with syringes from the caudal vein of groupers and allowed to clot at room temperature for 2 h. Serum was separated by centrifugation.

Phagocytic Activity

The number of cells was adjusted to 10^7 cells ml^{-1} in RPMI 1640 medium containing 10 % grouper serum (GS) using haemocytometer. The cells were allowed to adhere to a glass cover-slip (22 × 22 mm) for 1 h after which non-adherent cells were removed by washing with the medium. The phagocytic activity of grouper kidney leucocytes was examined as described by Yoshida *et al.* (1993).

Latex particles (0.85 ìm, 10^9 particles ml^{-1}, Difco, USA) were suspended in RPMI 1640 medium (10 % GS) and added to the cover slip and incubated for 2 h at 20 °C. Then, the cover slips were picked up using forceps and washed with the medium for 1 min. Cells were fixed with methyl alcohol, air-dried and stained with Giemsa. The number of adhered cells was about 5×10^5 cells cover $slip^{-1}$ and the number of phagocytic cells per 300 adhered cells was counted microscopically. The phagocytic activity (PA) was determined using the formula:

$$PA = \frac{\text{Number of phagocytizing cells}}{\text{Number of total cells}} \times 100$$

Determination of Antibody Titres

The antibody titres were determined based on the method described by Carpenter (1975). The serial of serum dilution was made as follows; 50 µl Phosphate Buffer Saline (PBS) were added to the well-1 up to well-12 of the microtitre plates. In the first well, 50 µl of serum were added and homogenized. Fifty µl of serum from the first well were taken and added to the second well and the dilutions were continued up to well-12. Fifty µl of *A.salmonicida* antigen were added to the well 1-12 of the microtitre plates and were mixed and homogenized, then incubated for 5–10 minutes. The antibody titre was indicated by agglutination on the highest serum dilution while there was no agglutination on the next dilution and measured as Serum Agglutination Unit (SAU).

Bacterial Challenge

Aeromonas salmonicida was used in the infectivity studies. *Aeromonas salmonicida* was cultured for 2 days at 25 °C on trypticase soy agar (TSA). At 29 days post-vaccination, the groups of control, vitamin C-treated fish and group of vaccinates were intramuscular (i.m.) challenged using 0.1 ml of 3×10^7 cells ml^{-1} suspended in saline and injected i.m and monitored daily for clinical signs and mortality for 14 days. Relative percent survival (RPS) of control, vitamin C-treated fish and vaccinated fish was determined over 14-day periods.

Statistical Analysis

Differences between groups will be examined by one-way analysis of variance (ANOVA) and Tukey's multiple comparison test.

RESULTS

Phagocytic Activity

The phagocytic activity of the kidney leucocytes from grouper treated with vitamin C were significantly higher than the leucocytes from control fish at 7, 14, 28 and 36 days after treatments (Table 1). The maximum stimulation of phagocytic cells was demonstrated in the leucocytes of fish treated with vitamin C at 28 days post-treatments.

Determination of Antibodi Titres

The antibody titre was started to be detected at 36 days post-treatments (1 week after bacterial challenge) and increased at day 42 in the grouper treated with vitamin C and *A.salmonicida* vaccine. Furthermore, a significant different of the antibody titre was found between control fish and the treated fish at 42 days after treatments (Table 2).

Table 1. The phagocytic activity in the leucocytes of groupers treated with vitamin C and *A.salmonicida* vaccine

Treatments	Days					
	7	14	21	28	36	42
Control (A)	5,30 ± 0,44^{a}	5,80 ± 0,46^{a}	6,53 ± 0,32^{a}	7,20 ± 0,20^{a}	6,30 ± 0,26^{a}	6,03 ± 0,15^{a}
Vitamin (B)	6,03 ± 0,32^{ab}	6,73 ± 0, 21^{ab}	7,33 ± 0,25^{ab}	8,60 ± 1,00^{ab}	7,50 ± 0,36^{ab}	7,03 ± 0,25^{ab}

The same letter (a or b) indicates no significant differences between treatments (P > 0.05)

Effects of Vitamin C and *A.salmonicida* Vaccine on Disease Resistance

The groups of fish treated with *A.salmonicida* vaccine showed higher RPS (90%) than control groups (53.3%) after challenge with *Aeromonas salmonicida*. Similarly, the groups treated with vitamin C showed higher RPS (80%) than the control groups following injection of *Aeromonas salmonicida* (Table 3).

DISCUSSION

An immunostimulant is a substance or action, which causes an innate immune response or increases an adaptive immune response. For the past ten years the research of immunostimulant in fish has gained increased acceptance because the wide range of parasites, fungi, bacteria and virus that affect the fish production, causing economic losses (Anderson, 1992). Recently, Sakai (1999) reviewed the current status of research into the use of immunostimulants in fish. Mainly, substances such as glucan, chitin, lactoferrin, and levamisole, as well as nutritional factors like vitamins B and C, growth hormone and prolactin are immunostimulatory because of their direct positive influence on innate immune elements such as phagocytic cell activity, natural killer cell activity, lysozyme levels and total immunoglobulin (Ig) levels. However, some of the immunostimulants could not be used because of various disadvantages, such as high cost, limited effectiveness upon parenterally administration, etc.

Vitamin C have been shown to increase phagocytosis in Rainbow trout (Blazer, 1982), Turbot (Roberts *et al.*, 1995) and Bagrid catfish (Anbarasu & Chandran, 2001). The results showed that vitamin C enhanced phagocytic activity in head kidney leucocytes of grouper 7, 14, 28 and 36 days after treatments. However, the phagocytic activity decreased after challenged with *A.salmonicida* (36 and 42 days post-treatments). This might be caused by the decreased number of neutrophil cells.

Antibody titre in the serum of grouper was 0.186 - 0.270 SAU when vitamin C was added, while when *A.salmonicida* vaccine was added antibody titre was 0.307 – 0.613 SAU. Antibody titre could be detected at 36 days after treatments, one week after *A.salmonicida* challenge, and increased at day 42. Phagocytosis initiates non-specific immune responses leading to specific immune responses by secreting antibody (Walczak, 1985). Vitamin C stimulated antibody responses to *E.ictaluri* in Channel catfish (Li and Lovell, 1985).

Vitamin C and *A.salmonicida* vaccine enhanced Relative Percent Survival (RPS) of grouper. At day 42, Relative Percent Survival (RPS) for control group was 53.3 %, vitamin C-treated group was 80.0 % and vaccinated group was 90.0 %. Vitamin C has been shown to increase

Table 2. The antibody titre in the serum (Serum Agglutination Unit) of groupers treated with vitamin C and *A.salmonicida* vaccine

Treatments	Days					
	7	14	21	28	36	42
Control (A)	0	0	0	0	$0,186^{a} \pm 0.08$	$0,270^{a} \pm 0,06$
Vitamin (B)	0	0	0	0	$0,236^{a} \pm 0.07$	$0,453^{b} \pm 0,08$
Vaccine (C)	0	0	0	0	$0,306^{a} \pm 0,04$	$0,613^{b} \pm 0,12$

The same letter (a or b) indicates no significant differences between treatments (P > 0.05)

Table 3. The Relative Percent Survival (RPS) of vitamin C and *A.salmonicida* vaccine-treated groupers challenged by *A.salmonicida*.

Treatments	Days					
	7	14	21	28	36	42
Control (A)	100	90	86,6	86,67	76,6	$53, 3^{a}$
Vitamin (B)	100	93	93,3	80	66,7	$80, 0^{b}$
Vaccine (C)	100	100	100	100	93,3	$90, 0^{b}$

The same letter (a or b) indicates no significant differences between treatments (P > 0.05)

disease resistance to *Enteric septicaemia* in Channel catfish (Liu et al., 1989), *Infectious hepatic necrosis* (IHN) in Rainbow trout (Anggawati-S *et al.*, 1989) and *Vibriosis* in grouper (Lin and Shiau, 2005). Bacterial infections caused by Gram-negative bacteria such as *Vibrio* sp., *Aeromonas* sp., and *Yersinia* sp. have been effectively controlled by vaccination.

The results of this study indicate that bath immersion of vitamin C provided an increasing of phagocytic activity (non-specific immune responses), titre antibody (specific immune responses) and protection against *A.salmonicida* infection in grouper. *A.salmonicida* vaccine also enhanced titre antibody and protection against *A.salmonicida* infection in grouper.

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