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Specific humoral immune response and protection against *Vibrio parahaemolyticus* in orange-spotted grouper *Epinephelus coioides*

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Abstract: This study shows the specific antibody response of orange-spotted grouper *Epinephelus coioides* to the formalin-killed bacteria *Vibrio parahaemolyticus* and the protection after experimental challenge with the bacteria. The vaccination was performed by intraperitoneal injection using bacterin combined with adjuvant. Specific antibodies were analyzed by ELISA using whole bacteria as antigen, and the immunoreactivity to the various bacteria protein components were detected by western blotting post vaccination. In addition, the protective efficacy of the bacterin was tested by challenge with *V. parahaemolyticus* at 30 and 60 days post vaccination. The results showed that significantly high level of antibodies was found in vaccinated fish using a bacterin with adjuvant. At 60 days post injection, the antibody levels were lower and more similar in all groups. By western blotting, immunoreactive bands were observed in bacterin vaccinated whereas no bands were seen in the two control groups. The challenge tests showed that the cumulative mortality rate was low in the vaccinated groups (10 and 40%) after 30 days post vaccination while high mortality occurred in control groups (70% and 90%). However, the mortality rate had increased in all groups at day 60 post vaccination. The bacterin/adjuvant vaccinated groups showed lowest mortality of 40% and the other groups including controls above 60%. Vaccination using formalin killed bacteria *V. parahaemolyticus* mixed with adjuvant FIA provided good protection in cultured *E. coioides.* This was supported by findings of specific antibodies to the bacteria in sera from vaccinated fish. The results indicate that vaccination, using adjuvants can be a prophylactic measure in orange spotted grouper and further studies should focus on obtaining longer time protection and use of various adjuvants.

Key Words: Antibody response, orrange-spotted grouper, *Vibrio parahaemolyticus*, vaccine, challenge

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Introduction

The grouper farming industry has rapidly expended in the recent decades and brought much needed revenue to Asian countries such as China, Indonesia, Malaysia, Taiwan, Hong Kong, Thailand, Philippine (Harikrishnan *et al.*, 2010). In Viet Nam, traditional grouper farming started in the early 90's in some provinces of the North and Center of Viet Nam (Le, 2004). However, as culture expanded, the fish farming industry experienced a variety of disease problems including viral diseases (Fukuda *et al.*, 1996; Hegde *et al.*, 2002; Lin *et al.*, 2007), parasitic diseases (Leong and Wong, 1988; Cruz-Lacierda *et al.*, 2001) and bacterial diseases (Ong, 1988; Saeed, 1995; Harikrishnan *et al.*, 2010).

Vibrio bacteria occur widely in aquatic environment and are part of the normal flora of coastal seawater and are opportunistic pathogens in many marine animals (Austin and Austin, 2007). Vibriosis is one of the serious bacterial diseases and it has been shown to occur worldwide where infections is reported from approximately 48 species of marine fish (Austin and Austin, 2007). The causative agents of vibriosis in grouper was described as *Vibrio alginolyticus* (Lee, 1995), *V. carchariae* (Yii *et al.*, 1997), *V. parahaemolyticus* (Danayadol, 1999; Najiah *et al*., 2003). The infections display lethargy, gastroenterisis, extensive haemorrhagic septicemia and ulceration of skin, fins and tail. Up to now, a few studies have been performed on immune system and vaccine for grouper to obtain protection to virus diseases (Lin *et al.*, 2007), parasites (Luo *et al.*, 2007) and bacterial infection (Harikrishnan *et al.*, 2012).

For marine cultured species *V. parahaemolyticus* has been reported to be pathogenic for abalones causing withering syndrome (Cai *et al.*, 2007), shrimp (Haldari *et al.*, 2007), and oyster (Yoon *et al.*, 2008). For marine fish, it caused high mortality of silver sea bream, *Sparus sarba* (Li *et al.* (1999), *Solea senegalensis* (Zorrilla *et al.*, 2003) and Iberian toothcarp, *Aphanius iberus* (Alcaide *et al.*, 1999), and the infection cause haemorrhagic and skin ulcer. In groupers, *V. parahaemolyticus* has caused disease in cultured fish in Thailand (Danayadol, 1999), Malaysia (Najiah *et al.*, 2003), China (Li *et al.*, 2010), Viet Nam (Do *et al.*, 2008; Nguyen and Nguyen, 2008). Diseased fish display lethargy, gastroenterisis, extensive haemorrhagic septicemia and ulceration of skin, fins and tail, resulting in high mortalities and economic losses.

Many kinds of antibiotic drugs have been used to control the diseases. However, the extensive use of antibiotic drugs and disinfectants for disease treatment and prevention has caused evolution of resistant bacterial strains to the drugs used. In addition the abuse

has resulted in residual drug components in the fish product, which is food for human consumption, and also to spread of drugs to the environment. Therefore, vaccination of fish is an important prophylactic measure to prevent disease in farmed fish.

The aims of this study were to measure the specific antibody response and protective efficacy obtained in vaccinated grouper using inactivated *V. parahaemolyticus* combined with adjuvant.

Materials and Methods

Bacterial strain and antigen preparation

Bacterial strain *Vibrio parahaemolyticus (isolate* V3) was isolated from kidney of diseased grouper in Viet Nam. Bacteria were cultured in 50 ml Tryptic Soy Broth (TSB, Difco) supplemented with 2% NaCl at 33°C for 24h, inactivated in 0.5% (v/v) formalin in 24h at 4°C (Aakre *et al.,*1994) and were tested for growth of bacteria. Then the suspension was centrifuged at 6,000 rpm for 10 min at 4°C and washed 3 times in PBS (Phosphate buffered saline, pH 7.4) to remove formalin. Bacterins were resuspended in PBS. Half of the bacterial suspension was mixed 1:1 (v/v) in Freund's incomplete adjuvant (FIA, Difco) to a bacterial concentration corresponding to 10^9 CFU/mL and the other half of suspension was added PBS (1:1). The type strain ATCC 17802 was used as reference strain.

Fish vaccination

Orange-spotted grouper juveniles *(Epinephelus coioides)* purchased from a private farm in Khanh Hoa Provinces, Viet Nam, were acclimated indoors in a 2-ton tank with seawater flow rate 0.5 liter per minute and fed twice daily with commercial pellet feed (NRD G16, INVE-Thailand Ltd) for 2 weeks. Fish with body weight and length of 49.8 ± 12.8 g and 14.7 ± 1.3 cm (mean ± SD), respectively, were randomly divided into 8 groups of 50 fish in 250 L. tanks. Treatments performed in duplicate included antigen with adjuvant (A), antigen without adjuvant (NA) and control groups (PBS-C and C, see below). Fish (N=50 per group) were vaccinated by i.p. injection with 0.1 mL bacterin suspension /fish and the same volume of PBS was used to i.p. injection for positive control group (PBS-C) and no injection for negative control group (C). All fish were starved for 24h and anaesthetized in ethylene monoglyco lether prior to injection.

Sampling of sera

Blood from the caudal vein of five random fish from each treatment was sampled at 30, 45 and 60 days post vaccination. Blood was coagulated at 4°C for 9h, centrifuged at 13,000 rpm for 15 min and the serum fraction was stored in aliquots at -70°C for further study.

Analysis protein profile

Antigen protein profile analysis was carried

out following SDS-PAGE method of Laemmli (1970): 5µg sample of *V. parahaemolyticus* per well were stacked in 5% (w/v) acrylamide gel, 12% (w/v) and separated using a Mini-Protein tetra cell (Bio-Rad) at 200 V for 35 min with low range prestained SDS-PAGE standards (cat. 161-0305, Bio-rad). Gels were silver stained according to Switzer *et al.* (1979) for analyses of protein profile.

Immunodetection

Protein profile of bacterial whole cells (WB) was separated by SDS-PAGE as described above. Immunodetection was performed using a Mini Transblot cell (Bio-Rad) by Western Blot. WB was transferred to NC (Pure Nitrocellulose Membrane 0.45 µm, cat. 162-0145, Bio-rad) at 100V/2h/4°C. The NC membrane was blocked in TBS (150mM NaCl, 20mM Tris base, pH 7.4) with 5% skim milk (v/v) for 1h at room temprature, then washed 3 times in TTBS (TBS and 0,5% Tween 20), and incubated in grouper sera (1:50/TBS) at 33°C for 2h. Bound grouper antibodies were detected by incubation with rabbit sera anti- grouper IgM (1:500/TBS) (kindly provided by the Fish Immunology group, University of Bergen, Norway) for 2h at 33°C. The membrane was then washed 3 times, followed by incubation with goat anti-rabbit IgG conjugated with horse-radish peroxidase (HRP, cat. 170-6515, Bio-rad) (1:3,000/TBS) for 1h at room temperature. Immuno reactive bands were visualised after incubation with HRP colour development reagent (cat. 170-6431, Bio-rad) for 30 min.

Antibodies titer

Specific antibodies in fish sera were analyzed by ELISA as previously described by Aakre *et al.* (1994). Freeze-dried sample of inactivated WB was dissolved in PBS (pH 7.3) and sonicated at 20kHz two times for 1 min. Microplates (96 wells, Nunc MaxiSorp^{TM}) were coated with WB/PBS (5µg/mL) (150 µl/well), incubated at 4°C overnight and washed 3 times in PBS-T (PBS with 0,5% Tween 20). Blocking of the microplates was performed by incubation in 3% skimmed milk/PBS-T for 1h at room temperature. Plates were then incubated with grouper sera (1:200/PBS-T, 100 µl/well) at 33°C for 2h and left overnight at 4°C. Bound grouper antibodies were detected by incubation with rabbit anti-grouper IgM (1: 3000; 50 µl/well) for 2h at 33°C and followed by incubation with goat anti-rabbit IgG conjugated with HRP (1:2000; 50 µl/well) for 1h at 33°C. The color was developed with O-phenylenediamine (OPD) for 10 min and the reaction was stopped with 2.0 M H_2SO_4. The antibody values were measured by iMark microplate reader (Bio-rad) at optical density (OD) of 490nm.

Challenge test and calculation of relative percent survival (RPS)

After 30 and 60 days post vaccination, 10

fish for each replicate were randomly selected to challenge by intramuscular (i.m.) injection of 0.2 mL V*. parahaemolyticus* at 1 x 10^8 cfu/mL.

The fish were then monitored daily for clinical signs and/or mortality over a 10- day-period. Dead fish were removed daily. Kidneys, livers and muscle lesions of moribund fish were sampled and used for isolation of bacteria. The mean percent cumulative mortality of challenged fish was determined for each trial during a period of 10 days. The relative percent survival (RPS) that considered as the protective efficacy was calculated according to Ellis (1988).

$$\textbf{RPS} = \frac{\%\ \text{mortality in vaccinated fish}}{\%\ \text{mortality in control fish}} \times 100$$

Statistical analysis

Antibody levels were compared by using ANOVA-one factor and Tukey HSD Post hoc test on SPSS statistical software version 15.0 with significance level of $P < 0.05$.

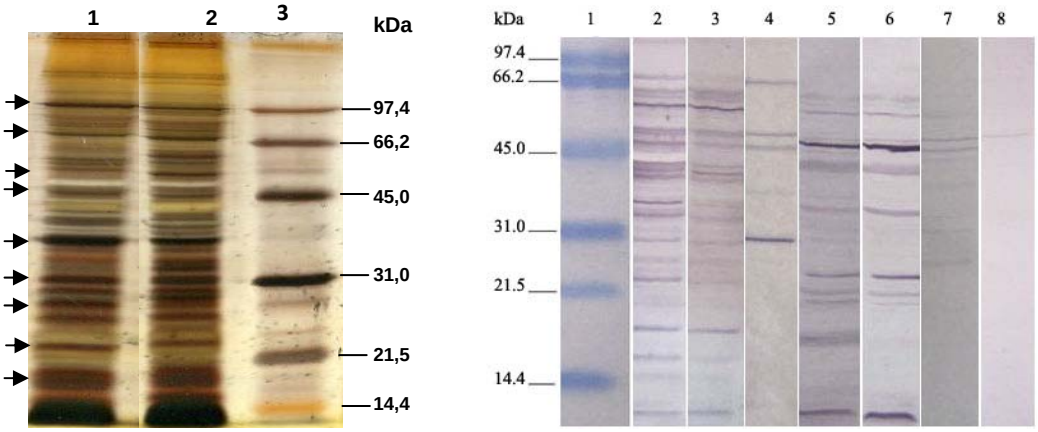
Results

Specific antibodies

The protein profiles of WB, strain *V. parahaemolyticus* and type strain ATCC 17802, are shown in Figure 1A. The specific immuno-reactions in immunoblot for vaccinated grouper sera to antigens from bacteria are shown in Figure 1B.

The protein profiles of strain *V. paraha-emolyticus* V3 and type strain ATCC 17802 in SDS-PAGE showed almost similar protein profiles with some major bands of molecular weight (MW) approximate 97.4, 66, 55, 47, 38, 33, 31, 27, 21, 18, 16, 14.4 and lower MW than 14.4 kDa (Fig.1A). Immunoreaction of antibodies to almost WB antigens following by immunoreactive bands was observed in all sera from vaccinated grouper while only one weak immunoreactive band in the control sera (Fig. 1B). Particularly, the strongest immunoreaction with the most immunoreactive bands was found in sera from group A at 30 days post vaccination. Then, the immunoreaction tended to be weaker at 45 and 60 days post vaccination expressing by less and weak bands (Fig. 1B, lane 2, 3, 4, respectively). Similar trend was found in group NA (vaccinated without adjuvant) (Fig. 1B, lane 5, 6 and 7 respectively). In addition, more immunoreactive bands were observed in sera from group A compared with sera from group NA for samplings analysed at all timepoints. The most frequent bands were seen at about 55, 47, 38, 33, 31, 28, 21, 18 and 14.4 kDa.

The antibody levels from fish sera at 30, 45 and 60 days post vaccination were analyzed by ELISA and the result showed differences among the groups (Fig. 2). Significantly high level of antibodies was found in group A, followed by group NA and the lowest level in the control groups after 30 and 45 days post vaccination.



A. **B.**

Fig. 1: a) SDS-PAGE profiles of the total protein of *Vibrio parahaemolyticus* isolateV3 (lane 1) and type strain ATCC17802 (lane 2) and molecular weight (MW) marker (lane 3). B) Immunoblots of individual representative grouper sera, using WB as antigen (lane 1: MW marker; lanes 2,3 and 4:bacterin with adjuvant group at 30, 45 and 60 days post vaccination; lanes 5,6 and 7: bacterin without adjuvant group at 30, 45 and 60 days post vaccination, respectively; lane 8: control serum)

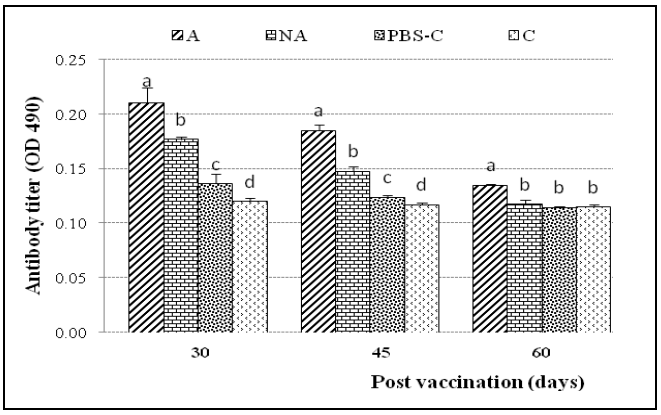


Fig. 2: ELISA antibody levels in sera from grouper *Epinephelus coioides* (serum dilution 1:200, n=5) Different letters (a, b, c, d) indicated significant differences among groups (p<0.05. Fish vaccinated with A: bacterin with adjuvant; NA: bacterin without adjuvant; PBS-C: control with PBS injection; C: control without PBS injection.

However, at 60 days post vaccination only the grouper injected bacterin with adjuvant showed elevated antibody level. In addition, the antibody levels were lower at the end of the test period for vaccinated groups. The levels were low in the control groups throughout the

experimental period.

Protection of vaccinated grouper after challenge with *V. parahaemolyticus*

The protective efficacy of vaccinated grouper after infection with *V. parahaemolyticus* was demonstrated by the higher survival of vaccinated fish compared with the nonvaccinated control fish (PBS-C and C) (Fig.3). The cumulative mortality of all groups were shown in Table 1.

The vaccinated groups showed a clear protection against *V. parahaemolyticus* with cumulative mortality at 10% (with adjuvant group-A) and 40% (without adjuvant group-NA), whereas high mortality observed in control groups (70-90%) (Fig.3a). RPS reached from 50 % (NA group) to 87.5% (A group) (Table 2). However, the survival was reduced in all vaccinated groups when challenged 60 days post vaccination (Fig. 3b), and the recorded RPS (Table 2) was 10.9% in the NA group and 41.1% in the A group. The symptoms of haemorrhagic septicemia and ulceration of skin were observed in diseased fish and *V. parahaemolyticus* were isolated from kidney and liver of moribund fish.

Table 1: The cumulative mortality of grouper *Epinephelus coioides*.

Challenge (day post infection)	Cumulative mortality %			
	With adjuvant (A)	Without adjuvant (NA)	PBS-C	C
30	10.0	40.0	70.0	90.0
60	41.2	62.4	65.0	75.0

Table 2: Relative percent survival (%) of immunised grouper *Epinephelus coioides*.

Challenging time (day post infection)	Treatments	
	With adjuvant	Without adjuvant
30	87.5	50.0
60	41.1	10.9

Discussion

In this study vaccinated and non vaccinated grouper were challenged with *V. parahaemolyticus* and the specific immune response after vaccination was analyzed.

Only few vaccination studies on *E. coioides* have been performed. These include vaccination

against virus diseases like viral nervous necrosis (VNN) resulting in a RPS of 64.2-69.5% with the highest antibody titers against VNN (Lin *et al.*, 2007), bath vaccination by formalin-inactivated- betanovirus which provided RPS of 39-43% (Harikrishnan *et al.*, 2010) and vaccination against a parasite by i.p injection with formalin kill *Cryptocaryon irritans* (Yambot and Song, 2006), all showing that protection could be obtained.

For marine fish, vaccines containing whole bacteria and a variety of bacterial components have been used. Although immunogenic, few vaccine are commercially available (Harikrishnan *et al.*, 2011) for farmed fish in Asia and so far none to grouper. Therefore, identification of a bacterium that could stimulate to production of protective immunity, would be of high interest. It is well known that the outer membrane protein (OMP) from some Gram negative pathogenic bacteria can induce protective immunity in fish (Kawai *et al.*, 2004; Li *et al.*, 2010; Harikrishnan *et al.*, 2011).

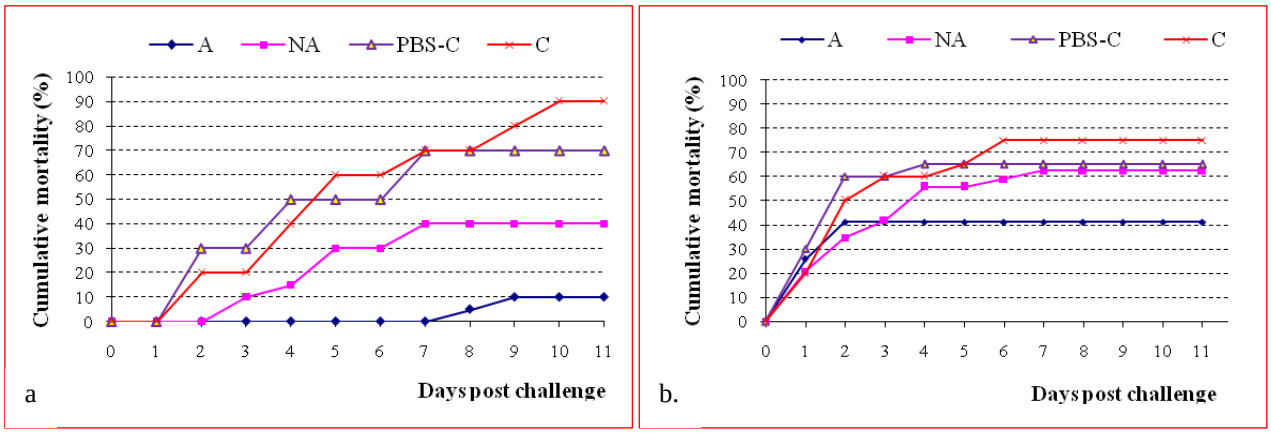


Fig. 3: Cumulative mortality of grouper *Epinephelus coioides* after challenge with *Vibrio parahaemolyticus* at 30 days (a) and 60 days (b) post vaccination (A: bacterin with adjuvant; NA: bacterin without adjuvant; PBS-C: control with PBS injection; C: control without PBS injection).

The present study showed that specific antibody levels in sera from vaccinated grouper to *V. parahaemolyticus* had been induced. The highest levels were found in vaccine containing adjuvant. This was confirmed by the immune-oblot analyses, showing the reactivity of induced antibodies to the various whole bacterial antigens. The immunoblots using vaccinated grouper sera with *V. paraha-emolyticus* as antigen showed that the most

frequent bands were found at about 55, 47, 38, 33, 31, 28, 21, 18 and 14.4 kDa, while only one weak band appeared in the control groups. This showed that the bacterial proteins were highly immunogenic and likely important *V. parahaemolyticus* antigens for induction of protective immunity. Recently, similar results have been found for *V. parahaemolyticus,* where five OMP (MW of 27, 32, 33, 47 and 48 kDa), provided high protective immunity in large yellow croaker, *Pseudosciaena crocea* (Mao *et al.*, 2007). Another study about OMP revealed that MW of 28 and 31kDa from *V. harveyi, V. alginolitycus, V. parahaemolyticus* induced protection in grouper *E. coioides* against infection by the corresponding pathogens (Li *et al.*, 2010). Based on these results it was recommended that the OMPs should be considered as vaccine components for the prevention of vibriosis infection.

By challenge of grouper with bacteria, the present study showed that the bacterin with adjuvant (A group) had higher protective efficacy compared to the bacterin without adjuvant (NA group). This was in accordance with the antibody levels, as group A had higher levels than the group NA and it also corresponded with more and stronger immuno reactive bands seen in Western blotting in sera from bacterin vaccinated fish (group A). Based on this, it is likely that the bacterin with adjuvant FIA stimulated grouper to produce antibodies mediating protection to *V. parahaemolyticus*. Adjuvants have been used since the 1920s to enhance the efficiency of vaccines administered to human and animals by acting as non-specific stimulators of the immune system and some cause the antigen to be released over a long period of time resulting in longer duration of the antibody response (Fodey *et al.*, 2008). Up to now, various adjuvants have successfully been implemented to commercial fish vaccines, especially to salmonids (www.pharmaq.no).

The duration of specific antibodies and protection was analyzed at three time points post vaccination. The cumulative mortality of grouper was low in fish vaccinated *V. parahaemolyticus*, but the RPS was reduced 60 days post vaccination. This corresponded to the lower antibody levels observed 60 days post vaccination. A similar study has been carried out on grouper *E. coioides* in Thailand showing that i.p. injection of vaccine using formalin inactivated *V. parahaemolyticus* provided the highest protection against bacterial infection with RPS value up to 77.6% (Clark *et al.*, 2010). Formalin inactivated bacteria also gave a protection efficacy in large yellow croaker, *Pseudosciaena crocea* with RPS up to 99% at 4 weeks after vaccination (Mao *et al.*, 2007). In addition, challenge test with *V. parahaemolyticus* on grouper *E. coioides* at 28 days post vaccination with OmpK as antigen showed mortality of 40% compared to 80% in the control group (Li *et al.*, 2010). The variable

protection has been observed in these studies support the present findings in that *V. parahaemolyticus* antigen can provide protection and be a relevant candidate in a fish vaccine.

In summary, high protection was obtained by vaccination with *V. parahaemolyticus* bacterin containing adjuvant FIA in *E. coioides*. This protective efficacy correlated with specific antibodies to various bacterial components and the antibody levels in the test groups. Based on these results, it should be possible to use vaccination as prophylactic measure to motality in orange-spotted grouper caused by *Vibrio* pathogens.

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