

**THE ADMINISTRATION OF MIXTURE *Vibrio parahaemolyticus* AND *Vibrio alginolyticus*
BACTERIN AND THEIR COMPONENTS TO ENHANCE THE SURVIVAL RATE OF SHRIMP
*Penaeus monodon***

PEMBERIAN CAMPURAN BAKTERIN DAN TURUNANNYA DARI *VIBRIO* *Vibrio parahaemolyticus*
DAN *Vibrio alginolyticus* UNTUK MENGUATKAN SINTASAN UDANG *Penaeus monodon*

Hari Suprpto ¹

¹ Department of Fisheries, Faculty of Veterinary Medicine, Airlangga University
Mulyorejo Kampus C Surabaya 60115 Indonesia Tel/Fax. 031-599-3015

ABSTRACT

The vibriosis in hatchery and farm easily recognized by the luminous the bacteria present in the water and shrimp itself. The *Vibrio* sp. used in this experiment were isolated from shrimp and rearing water and the LD₅₀ of *V. parahaemolyticus* is 10^{2.6} and *V. alginolyticus* is 10^{2.5} CFU/fish. The best results obtained from 10 mg/ml mixed of both *V. parahaemolyticus* and *V. alginolyticus*. The protection of OMP vaccinated fishes was relatively low, the reason could be the OMP contained the low antigenic substance. The SR of fish vaccinated FKC and ICC is 65-70 percent 70-75 percent. Even though it was not proved that FKC and ICC contents more antigenic substance, the FKC and ICC have the higher protein than OMP in measurement of protein and electrophoresis.

Key words : *V. parahaemolyticus*, *V. alginolyticus*, Lipopolysaccharide, Intracellular components, Outer membrane protein, Formalin killed cells

ABSTRAK

Vibriosis di panti pembenihan dan tambak dapat dikenali dengan mudah dengan adanya bakteri yang bersinar ada didalam air dan udang. *Vibrio* sp. yang dipakai di dalam penelitian ini diisolasi dari udang dan tambak dan LD₅₀ dari *V. parahaemolyticus* adalah 10^{2.6} dan *V. alginolyticus* adalah 10^{2.5} CFU/fish. Hasil yang terbaik didapatkan dari dosis 10mg/ml baik dari *V. parahaemolyticus* dan *V. alginolyticus* Proteksi dari udang yang divaksin dengan OMP rendah karena OMP mengandung sedikit substansi antigenik. Sintasan dari ikan yang divaksin dengan FKC dan ICC adalah 65-70 percent dan 70-75 persen. Walaupun belum dibuktikan bahwa FKC dan ICC mengandung lebih banyak substansi antigenik, FKC dan ICC mempunyai kandungan protein yang lebih tinggi dibandingkan dengan OMP diukur dari kandungan protein dan elektrophoresis.

Kata kunci : *V. parahaemolyticus*, *V. alginolyticus*, Lipopolysaccharide, Intracellular components, Outer membrane protein, Formalin killed cells

INTRODUCTION

The tiger shrimp *Penaeus monodon* industry have been growing fast in East Java Province, since 1990 because the tiger shrimp have a big size and superior price. Recently the shrimp industry infected by the bacteria and the significant bacterial disease is *Vibrio* sp. (Zafran *et al.*, 1998; Lavilla-Pitogo and de la Pena, 1998). The vibriosis occurred in shrimp industries especially in dry season when water quality tends to be deteriorate (Ellis, 1998). The bacteria are normal flora of freshwater and brackish water environment (Allen *et al.*, 1983) and the pathogenicity of *Vibrio* sp. in fish increase if the water polluted (Robert and Horne, 1978). Suprpto and Raharjo (2000) reported that vibriosis is caused by two strain of *V. parahaemolyticus* and *V. alginolyticus*. Furthermore Tanasomvang *et al.*, (1998) reported that the flora of rearing water in shrimp hatcheries was comprised mainly of *Vibrio* spp. and these bacteria were associated with mortalities of shrimp stocks. The main *Vibrio* in rearing shrimp water including *V. alginolyticus*, *V. damsela*, *V. cholera* (non o-1) *V. fluvialis*,

V. harveyi and *V. parahaemolyticus* (Ruangpan *et al.*, 1995). The economic loss due to the disease was well documented by the Directorate General of Fisheries (Direktorat Jenderal Perikanan, 1997). These loss attributed to bacteria and virus disease in both backscatter pond and hatcheries. Purpose of the research is to enhance survival rate (SR) of shrimp against *V. parahaemolyticus* and *V. alginolyticus* infection and to find the prophylaxis method against those bacterial infection.

MATERIALS AND METHODS

Shrimp and bacterial strains

The healthy tiger shrimp *Penaeus monodon* 10-20 g in body weight were purchased from a farms in Pasuruan. The shrimp acclimated in concrete tank and rapid diagnosis to check the health status of shrimp was conducted immediately. Shrimp were kept in rectangular aquarium (40 l) with aeration and fed with commercial feed during the course of experiments.

V. parahaemolyticus and *V. alginolyticus* strain used in this experiment were isolated from diseased of shrimp (Suprpto, 2000). Those bacteria were stored in tryptic soy broth (TSB, BBI Becton Dickinson and Co.) with 10 % glycerol at -80°C until used. Prior the

experiments, bacteria were sub cultured on nutrient agar (NA, Eiken) enriched with NaCl incubated at 25 °C for 48 h.

Preparation of Formalin-Killed Cells

Formalin Killed Cells (FKC) were prepared as previously described by Suprpto *et al.*, (1996), briefly, sterilized cellophane sheets were placed on NA plates and then precultured *V. parahaemolyticus* and *V. alginolyticus* were inoculated on each separate plate with sterilized glass rods. The bacteria were then incubated at 25°C for 48 h, harvested with 2 ml of 0.01 M phosphate buffered saline (PBS, pH 7.0). The cell suspension was centrifuged at 10,000 rpm for 20 min and resultant supernatant was disposed. The centrifuged cells added with formalin 0.5 % and incubated at 25°C for 72 h. The cells were washed three times with PBS and adjusted to 50 mg/ml consisted of 25 mg/ml of *V. parahaemolyticus* and 25 mg/ml of *V. alginolyticus* in PBS and stored at 4°C after sterility check, this cells was designed as mixture of FKC and proteint content was measured using Folin Phenol (Lowry *et al.*, 1951)

Preparation of Lipopolysaccharide

Lipopolysaccharide (LPS) was extracted with hot phenol water (Westphal and Jann, 1965), briefly the *V. parahaemolyticus* and *V. alginolyticus* were inoculated on sterilized cellophane sheets separately on each NA plates and then precultured bacteria were inoculated on each plate. After incubation at 25°C for 48 h, cells were harvested with 2 ml of 0.01 M phosphate buffered saline (PBS, pH 7.0) and the supernatant was disposed. Prior to extraction the cells adjusted to 5 % with PBS, heated in water bath at 68°C. The extracted solution A composed of 90% (w/v) phenol preheated to 68°C, mixed properly at 68°C for 20 min. Cells were removed by centrifugation at 10.000 rpm for 15 min. The resulted supernatant was dialyzed with tap water for overnight and lyophilized. Homogenized of lyophilized crude LPS mixed with extraction B solution with composition, A extraction solution: chloroform: light petroleum (2:5:8) with the volume as in A extraction step for 2-5 min was done in room temperature. The chloroform and petroleum were removed by rotary evaporator and followed by precipitation of LPS in drop wise addition of distilled water. Collection of precipitation by centrifuged at 100,000-x g for 3 h, the obtained pellet washed 1-2 times with water and lyophilized. The preparation of mixture LPS composed of both half LPS

extracted from *V. parahaemolyticus* and *V. alginolyticus*.

Preparation of Intracellular components

Bacterial pellet from the cellophane plate culture was washed three times with PBS, and cell concentration was adjusted to 50 mg/ml in PBS. The cells were sonicated for 20 min (Branson sonicator, Model 250), centrifuged at 12.000 rpm for 20 min, and filtered with a 0.45- μ m filter. The ICC composed of both half ICC extracted from *V. parahaemolyticus* and *V. alginolyticus*. and stored at 4 $^{\circ}$ C after sterility check.

Pathogenicity and toxicity tests

Groups consisting of 10 shrimp were injected intramuscularly with bacteria diluted 10-folds in PBS, prepared from 48-h cultures on NA (25 $^{\circ}$ C). Shrimp were injected with 0.2 ml bacterial suspensions at $10^2 - 10^5$ CFU/ml. Control shrimp received intramuscularly injections of 0.2 ml ml PBS, respectively. In toxicity tests, undiluted or serially 2 fold-diluted ECP or ICC were injected intramuscularly into shrimp (0.2 ml/fish). These injection doses and volumes per fish were determined from the results of preliminary experiments. After injection, fish were kept in flow-through 40 l plastic tanks (20 $^{\circ}$ C) with supplemental aeration, and mortalities were recorded daily for 2 weeks. The LD50 of bacteria were calculated according to Reed and Muench (1951). Bacterial isolations were attempted from the hepatopancreas of dead shrimp on NA at 25 $^{\circ}$ C.

Immunization of fish

Shrimp were immunized actively by two im-injections with mixture ECP, ICC, LPS (0.025-0.15 ml/fish) and FKC (5-15 mg/fish) of both *V. alginolyticus* and *Vibrio parahaemolyticus*. The control fish are separated in other tanks and injected only PBS as immunogens. The booster injections were administered after 7 days after first injection. One weeks after the booster injection, fish were im-challenged with live cells (10^3 CFU/fish) and observed at 20 $^{\circ}$ C for 2 weeks. The bacteria was isolated from the dead fish on NA at 25 $^{\circ}$ C for 48 h to confirm that mortalities were caused by the injected *V. parahaemolyticus* and *V. alginolyticus*.

Electrophoresis

SDS-PAGE analyses were carried out according to the method described by Laemli (1970). The solution of ECP, ICC, FKC and LPS were mixed with 0.17M Tris-HCl buffer (pH 6.8) containing SDS (5.3%) and 2-

mercaptoethanol (13.2%). Electrophoresis using 10% gel and Tris (0.025M) glycine (0.192M) buffer containing 0.1M SDS was performed at a constant volt of 150 for 1 h. The protein bands were stained with Coomassie brilliant blue.

RESULTS AND DISCUSSIONS

The LD50 of *V. parahaemolyticus* is $10^{2.6}$ CFU/fish and *V. alginolyticus* is $10^{2.5}$ CFU/fish. The dead of shrimp within range 4-10 days after the injection of bacteria to shrimp. The clinical signs of dead shrimp are the development of light red color in the whole body. Vaccination of shrimp with LPS showed 70-75 percent protection against live cells challenged, followed by FKC 65-70 percent, and both ICC and OMP 50-65 percent shrimp survived against live cells challenged. The results of vaccination with 4-vaccine preparation in shrimp were presented in tables 1-4. The vaccination of shrimp with ECP did not showed high protection against live cells challenged (data not presented). The protein contents of ECP relatively low than OMP and ICC, because the ECP is secreted by the bacteria and released into environment or/when bacteria lyses. The protein contents and molecular weight of vaccine preparation were presented in Figure 1 and 2. The OMP may content of low immunogenic protein because the SR of shrimp were relatively low than other preparation. The electrophoresis presented in Figure 1, showed that all protein band may have the protective antigen. The SR of shrimp has a correlation with protein content.

Aquaculture represent one of the fastest growing food producing sector in Indonesia, because the rapid growth of aquaculture over 1980. One of the fastest growing aquaculture production sector is penaeid shrimp. Total world shrimp production in 1999 accounted 14 percent of the total aquaculture production value 47.5 billion USD (FAO, 2001). Therefore the penaeid shrimp industry has develop into a major industry. The problem of Indonesia penaeids industry is emerging new disease and the bacterial disease leading by the *Vibrio* sp. One of the prophylactic method to overcome the disease is vaccination against the bacterial. Fish vaccination enhances the cellular immunity and humoral immunity. Increased Phagocyte Index of macrophage exhibits cellular immunity, and humoral immunity by increase the antibody titer of vaccinated fish. In human, macrophage has been characterized well, but in fish it was called granulocyte, because the function in defense mechanism

Table 1. The survival of vaccination regime of *P.monodon* with the mixed of formalin killed cells (FKC) of both bacteria and challenged with 2.0×10^3 cell/ml of the separate *V. parahaemolyticus* and *V. alginolyticus*, observed in 14 days kept at room temperature.

Doses of antigens	Percent survival of shrimp to <i>V. parahaemolyticus</i>	Percent survival of shrimp to <i>V. alginolyticus</i>	Control shrimp (only PBS)
5 mg/shrimp	35	40	10
10 mg/shrimp	65	70	10
15 mg/shrimp	60	55	10
20 mg/shrimp	40	40	10

Table 2. The survival of vaccination regime of *P.monodon* with mixed of ICC vaccine of *V. parahaemolyticus* and *V. alginolyticus* in 14 days kept at room temperature, challenge with 2.0×10^3 cell/ml.

Doses of antigens	Percent survival of shrimp to <i>V. parahaemolyticus</i>	Percent survival of shrimp to <i>V. alginolyticus</i>	Control shrimp (only PBS)
0.025 ml/shrimp	30	30	15
0.05 ml/shrimp	50	65	15
0.10 ml/shrimp	70	75	15
0.15 ml/shrimp	50	55	15

Table 3. The survival of vaccination regime of *P.monodon* with mixed LPS vaccine of *V. parahaemolyticus* and *V. alginolyticus* in 14 days kept at room temperature, challenge with 2.0×10^3 cell/ml.

Doses of antigens	Percent survival of shrimp to <i>V. parahaemolyticus</i>	Percent survival of shrimp to <i>V. alginolyticus</i>	Control shrimp (only PBS)
0.025 ml/shrimp	50	45	10
0.05 ml/shrimp	70	75	10
0.10 ml/shrimp	70	65	10
0.15 ml/shrimp	50	45	10

Table 4. The survival of vaccination regime of *P.monodon* with mixed the outer membrane protein vaccine of *V. parahaemolyticus* and *V. alginolyticus* in 14 days kept at room temperature, challenge with 2.0×10^3 cell/ml.

Dose of antigens	Percent survival of shrimp to <i>V. parahaemolyticus</i>	Percent survival of shrimp to <i>V. alginolyticus</i>	Control shrimp (only PBS)
25 mg/ml	35	30	15
50 mg/ml	50	65	15
75 mg/ml	55	55	15
100 mg/ml	40	45	15

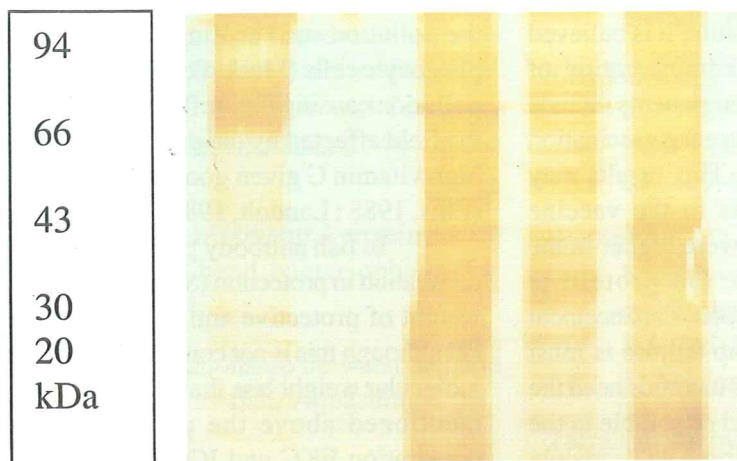


Figure 1. Electrophoresis mixture of *V. para-haemolyticus* and *V. alginolyticus*. Vaccine preparation. 1 : Marker, 2 : LPS 3 : FKC, 4 : ICC, 5: OMP.

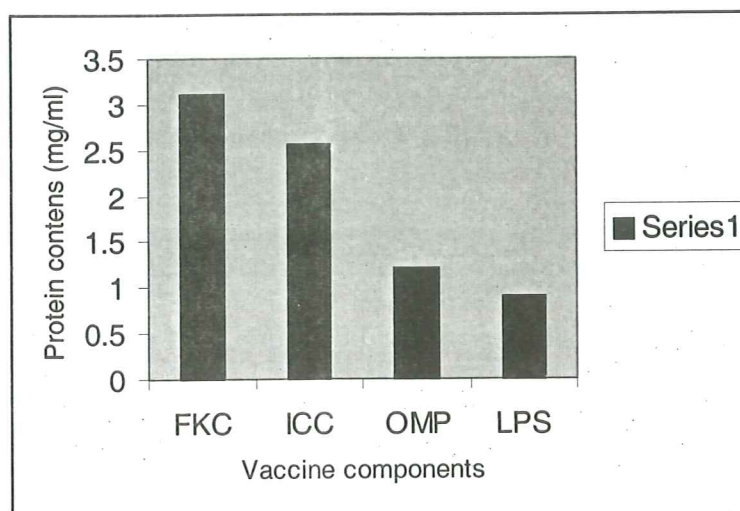


Figure 2. The protein concentration of 4-mixture vaccine preparation of *Vibrio* sp.

is not yet understood completely. The immunization of fish with FKC confer the protection against the live cells challenged, this attributed that the FKC may contain the high antigenic substances. The protein content of FKC was higher compared to other components vaccine such as ECP, ICC and LPS. It may also affected by the methods of delivery vaccine to shrimp. The injection is the best method to deliver vaccine into fish, other methods such as oral have a problem with digestion of antigen. Maximal protection of shrimp followed the administration of 10 mg/shrimp of antigens for 14 days, an important finding that high administered antigen did not causing the higher protection of vaccinated fish.

It was showed that SR of ECP vaccinated shrimp only less than 45 percent even shrimp have been injected the second time with the ECP. In the future it is believed that SR could be increased by the administration of bacterial and their components. The components include ICC present in the bacterial itself because the vaccination with the ICC exhibited good results. This results may caused by the high protein contents in the vaccine preparation. Protein contents of ICC were higher in the vaccine preparation but whether the protein is immunogenic or not it is not yet understood. Enhancement of the defense system in the of shrimp culture is most feasible by oral administration, but this methods need the research to prevent the antigens spread or soluble in the water. Immunostimulation of culture shrimp will certainly continue to play an important role in disease control in intensive shrimp culture.

Commercial vaccine in the Northern hemisphere contain mixture of the commonly encountered species which disease problems i.e. *V. anguillarum* and *V. ordalii*. The majority of the commercial vaccine sold is simple inactivated culture composed of mixture whole cells, extra cellular products. In the case of *V. anguillarum* the protective antigens appear to be lipopolysaccharide (LPS) in the cell wall. LPS of the bacteria were extracted and used for vaccination in shrimp are presented in Table 3, with the results almost equal to FKC and ICC.

To understand the immunogenic LPS it should be purify and injected to shrimp. In this vaccination LPS was not administered with adjuvant, shrimp just only injected with crude LPS and observed for 14 days. The vaccine are limited to use on average body weight 20 g in experimental vaccination, because the small size are not allowed to be injected, therefore in the future immersion or oral experiment must be conducted.

The electrophoresis results of vaccine preparation namely FKC, LPS, ICC and OMP showed that molecular weight of protective antigen estimated to be 5-200 kD, but which one the true protective antigen is not yet understood. The band of OMP is so thin because the low concentration of protein may present in the solution, but on the other hand band of FKC and ICC visualized clearly because the higher concentration of antigens. This results presented in this experiment were doing in the laboratory therefore in the field may slight different in the protection can be occurred, because some environmental stressor may really acting as predisposing factors in diseases.

Another important factor in this experiment because fish was not exposed to polluted water, because the pollution such as Zinc caused the lower activity of phagocyte cells (Mushiake, 1985), and some of the metal pollution causing fish suffered to anemia. Vaccination in the field affected by the use of feed, by feeding fish with high vitamin C given good protection in the experiment (Ellis, 1988 ; Landolt, 1989).

In fish antibody production is not always have a correlation to protection (Song and Kou, 1981). Molecular weight of protective antigen estimated to be 5 kD-120 kD although this is not confirm in this experiment because molecular weight less than 5 kD is not immunogenic. As mentioned above the protein contents of vaccine preparation FKC and ICC were high and followed by OMP, LPS. The protein concentration of five prepared vaccine were presented in Figure 2.

The protection of vaccine preparation has a correlation with the protein content, although the protection was different between the vaccine compositions. Even though the LPS have a low concentration of protein but the SR of fish relatively high, the reason could be LPS contain more immunogenic substance. The low concentration of immunogenic protein in LPS due to extraction of LPS with hot phenol water was not perfect because the light phenol smell present in the LPS vaccine preparation. The results of the experiment promising the development of the *Vibrio* vaccine in shrimp, but the vaccine need some modification.

REFERENCES

- Allen, D. A., Austin B. and Colwell R. R. . 1983. Numerical taxonomy of bacterial isolates associated with a freshwater fishery. *J. Gen. Microbiol.*, 129 : 2043-2062.
- Ellis, A.E. 1988. Fish Vaccination. Academic Press. Pp 1-50.
- Direktorat Jenderal Perikanan. 1977. Makalah Direktur Bina Program Pada Pertemuan Koordinasi dan Pemantapan Rekeyasa Teknologi Pembenuhan Lintas Unit Pelaksana Teknis Direktorat Jenderal Perikanan di Yogyakarta, 8-12 Juni 1997.
- FAO FishStat Plus. 2001. Food and Agriculture Organization of the United Nation. Fisheries Department Statistical Database and Software, Version 2.30. <http://www.fao.org>. (accessed December 2001).
- Laemli, U.K. 1970. Cleavage of structural protein during the assembly of the head bacteriophage T4. *Nature*. 227 : 680-684.
- Landolt, M.L. 1989. The relationship between diet and the immune response of fish. *Aquaculture*. 79 : 193-206.
- Lavilla-Pitogo, C.R. and de la Pena, L.D. 1998. Bacterial disease in shrimp *Penaeus monodon* culture in Philippine. *Fish Pathol.*, 405-410.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall R.J., 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chemistry* 193 : 265- 275.
- Mushiake, K., Nakai, T. and Muroga, K. 1985. Effects of Zinc on the activity of Eel *Anguilla japonica* leukocyte. *Fish Pathol.* 6 : 125-130.
- Reed, M and Mueench, R. 1958. The calculation of LD50. *J. Bio. Chemistry*. 5 : 25-27.
- Robert, R.J. and Horne M.T. 1978. Bacterial meningitis in farmed rainbow trout *Salmo gairdneri* Richardson, affected with chronic pancreatic necrosis. *J. Fish Dis.* 1 : 157-164.
- Ruangpan, L., Tabkaew, R. and Sangrungruang, K. 1995. Bacterial flora of ponds with different stocking densities of black tiger shrimp *Penaeus monodon*. In."Disease of Asian Aquaculture II". Eds by M.Shariff *et al.*, Fish Health Section., Asian Fish. Soc., Manila Pp141-149.
- Suprpto, H., Hara, T., Nakai, T. and Muroga, K. 1996. Purification of a lethal toxin of *Edwardsiella tarda*. *Fish Pathol.* 31 : 203-207.
- Suprpto, H. and Raharjo D., 2000. The isolation of bacterial pathogen from shrimp and their water in Bangil Pasuruan East Java Province. Proceeding National Seminar in UGM Yogyakarta, July 15. 2000.
- Song, Y. L. and G. H. Kou. 1981. The immune response of eel *Anguilla japonica* against *Edwardsiella anguillimortifera* as studied by immersion method. *Fish Pathol.*, 15, 249 - 255.
- Tanasomwang, V., Nakai, T., Nishimura, Y. and Muroga, K., 1998. *Vibrio*-inhibiting marine bacteria isolated from Black tiger shrimp hatchery. *Fish Pathol.* 33 : 459-466.
- Westphal, O and Jann, K., 1965. Bacterial lipopolysaccharides. Extraction with phenol-water and further application of procedure. In : Whistler R.L. (ed) *Methods in carbohydrate chemistry*, Vol 5, Academic Press, Inc. New York, Pp 83-91.
- Zafran, F. Johny, Rosa, D., Koesharyani, I. and Hatai, K., 1998. Increased survival of *Penaeus monodon* larvae treated with *Vibrio harveyi* bacterin. *Fish Pathol.*, 33 (4) : 449-450.