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In vivo Study of Immune Response against *Vibrio vulnificus* in *Mugil cephalus* by Vaccination

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ABSTRACT

Vibrio vulnificus isolated from diseased Mullet, *Mugil cephalus* L., were immunized with formalin inactivated *Vibrio vulnificus* to the juveniles of priming and booster groups of fish. Assessing the humoral immune response using the serum agglutination assays. This experiment was performed to compare the antibody titre in priming dose and booster dose. The result revealed that the higher priming response at 21 dpp and booster response at 35 dpb. This intramuscular injection was more suitable for inducing the primary response than intraperitoneal injection.

Key words: Immune response, *Vibrio vulnificus*, juveniles of *Mugil cephalus*, Agglutination titre, primary response, booster response.

INTRODUCTION

Aquaculture is growing rapidly world wide with fish being the primary source of animal protein in many countries. The exploitation of human population & the industrial development. Our ecosystem has highly polluted and causing considerable stress to fishes & other aquatic animals.

The occurrence of disease outbreaks in aquaculture system can be attributed to a number of reasons. The lack of understanding of intricate balance between the host, pathogen and the environment was most obvious. These conditions, causes the organisms to be more prone to infections. The information so generated, forms as the base line for the development of vaccine. Ogbulie (1998) studied the efficacy of chemotherapeutic agents in controlling vibriosis in cultured fishes. Antibiotics are frequently used to cure diseases but there is always a risk of bacteria developing resistance and residues in the product (Fjalestad *et al.,* 1993).

The development of drug resistance of fish pathogens has frequently been reported (Aoki, 1992). Virbriosis has been a major cause of concern especially in brackish water & marine aquaculture systems. *Vibrio vulnificus* is one of the fish pathogens in marine & brackish water (Thampuran *et al*., 1998). The first attempt of vaccination was provided by Hayashi *et al.,* (1964) preparing vaccine against *Vibrio.* Collado *et al*. (2000) studied the effectiveness of different vaccines for Vibriosis caused by *Vibrio vulnificus* bio type 2 in European eels. Commercial vaccines are available to prevent vibriosis. The various studies revealed that several substances have been tested for the prevention of bacterial diseases including whole cell bacteria (Fukuds & Kusuda, 1981), attenuated live vaccines (Kusuda and Hamaguchi, 1988),

Lipopolysaccharide extract (Fukuda and Kusuda, 1982; 1985). Liposomal vaccine (Kusuda *et al.,* 1988) and toxiod-enriched whole cell vaccine (Magarinos *et al.,* 1994) and they have not been very effective in aquaculture farms, despite being effective in laboratory trials.

In the present study vaccination of *Mugil cephalus* against *Vibrio* isolate and consequent analysis of several antibody titres were performed.

MATERIALS AND METHODS

Collection and maintenance of experimental animals

Juveniles (6-10 cm) of gray mullet (*Mugil cephalus* , Mugilidae) were collected from local water bodies in and around Muttukadu, CIBA, Chennai. They were maintained in large FRP(Fiberglass Reinforced Plastic) tanks of 500 litres capacity. During the period of study, the physio-chemical parameter of water, the temperature ranges from 27° ± 27.5° and pH 7.4 ± 7.4 were maintained properly.

Isolation of bacterium

The bacterium was isolated from naturally infected fishes. Infected moribund fishes were identified & samples drawn aseptically were used for bacterial cultures. The collected samples like blood, kidney tissues were inoculated in suitable culture medium such as Zobell's Marine agar, alkaline peptone water, & Thiosulfate Citrate Bile salt Sucrose (TCBS) agar. Further identification characters were done with Bergy's Determinative Bacteriology.

Preparation of vaccines

Formalin inactivated bacteria of *Vibrio vulnificus* were prepared by using standard method. Lillehang (1989), Culture inoculated alkaline peptone water was centrifuged at 10,000 rpm for 15 minutes & washed thrice by using sterile Phosphate buffer saline (PBS). The final pellet was resuspended in PBS to 10^{-9} CFU/ml. The cell density was enumerated by Standard Plate method. The bacterial suspension was then serially diluted to obtain 10^{-7}-10^{-9} CFU/ml. The suspensions were inactivated with 0.5% formalin for 24 hours. The inactivated suspensions were harvested by centrifugation as mentioned earlier.

Immunization of fish

The fishes were maintained in two groups viz., primed and booster for each dilution (10^{-6}, 10^{-7}, 10^{-8} CFU/ml) to evaluate the effect of different doses of the bacterium with primary & booster application. *Vibrio vulnificus* vaccines were administered by intra-muscular injection. For immunization, 36 fishes were taken for each dilution, with about 18 fishes for each groups in triplicates, i.e., Primed (6x3), Booster (6x3) were injected intramuscularly with 0.1 ml of bacterial suspension from respective dilution. The control fish group was injected intramuscularly with 0.1 ml / fish of sterile PBS. The water was changed everyday throughout experimental period. The three groups of fishes were sampled for serum antibody analysis at zero day post injection 0 dpi, 7 dpi, 14 dpi and 20 dpi respectively. A booster dose was given to the booster group of fishes at 21 dpi containing the same amount of bacterial suspension.

Agglutination titre

At 7 days intervals, starting from zero dpi, ten fishes were selected. The blood samples of *Mugil cephalus* were collected through caudal vein after anesthesia (Benzocaine, 10ppm). Blood was allowed to clot and stored at 4° overnight. The serum was separated by centrifugation at 6000 rpm for 10 minutes and inactivated at 50° for 30 minutes.

Sterile PBS (pH 7.2) was added (50ml) to each well of a 96 well 'U' bottom micro titre plate (Tarson India Ltd.). The 1st well was added with 100 ml of inactivated serum. From the 1st well 50ml of inactivated serum was transferred to the 2nd well .This serial dilution was continued till the 11th well. The last well without serum served as negative control. Later, inactivated *Vibrio vulnificus* suspension (10^{-8} CFU/ml) was added to each well (50ml) & incubated at room temperature for 1 hour & overnight at 4°C. The last dilution of serum showing clear agglutination was taken as the end point for titre estimation. Agglutination titres for each fish sample were expressed as $\log^2$ values based on visual observation (Sundick and Rose, 1980).

Statistical Analysis

The serum Agglutination titres were

subjected to ANOVA following Snedecor and Cochram (1968).

This is used to assessing the efficacy of application to evaluate the specific immunomemory (Nosal *et al*. 1965). The titre at the corresponding moment during the primary response. The formula is,

$$MF = \frac{S(X)\text{-}S(O)}{P(X)}$$

Where,

S (X) - titre at 'X' day after booster in the booster group

S (O) - titre at the day of booster in primed group and

P (X) - titre of the primed group at 'X' day after booster.

RESULTS AND DISCUSSION

The naturally infected juveniles of *Mugil cephalus* were sluggish and necrotic in nature. Most of the infected fishes had hemorrhagic spots, boils & blackening of body surface. All the primary culture medium produces corresponding colony morphologies. Biochemical reactions confirmed the species as *Vibrio vulnificus*.

Table 1and 3 showed the primary & booster immune responses of *Mugil cephalus* to formalin treated *Vibrio vulnificus*. The higher response in priming group injected with 10^{-8} CFU/ml at 21 dpp was 7.6 ± 0.55 (Antibody titre). There is a sharp rise in 14 days with stabilization in higher dose. After 21 days the primary response has dropped down to (5.4± 0.55). In booster immune response, the higher antibody titre at 35 dpb was 11.6 ± 0.55.

Table 2 and 4 showed the statistical analysis for ANOVA calculation. ANOVA for testing the difference between the doses and days after priming booster revealed highly significant difference between the groups. The statistical analysis revealed the significant (P >0.05) difference between the doses and durations of both primary and booster response in *Mugil cephalus* to intra-muscularlly injected *Vibrio vulnificus*.

The result revealed that a clear progressive positive response against the injection. It indicates that injection method, formalin treated antigen to the fishes recorded the higher titre on the 21 dpp and the secondary responses recorded the higher titre on 35 dpb respectively. However in the higher dose, the response after 14 days of post booster reached the peak and stabilized. Hung *et al.* (1997) demonstrated the effects of adjuvant and booster injection on antibody production in eels. The booster injection provoked higher antibody titre. This study also showed the level of the secondary antibody response were positively correlated with the primary dose. Usually the booster response reached a

Table 1: Immunization of *Mugil cephalus* with formalin inactivated *Vibrio vulnificus* (primary dose)

Dpp*	Dilution		
	Log^{-6}	Log^{-7}	Log^{-8}
0	0.6 ± 0.55	0.6 ± 0.55	0.8 ± 0.45
7dpp	3.0 ± 0.70	4.4 ± 0.55	5.8 ± 0.45
14dpp	4.6 ± 0.55	5.2 ± 0.45	7.4 ± 0.55
21 dpp	6.2 ± 0.45	6.6 ± 0.55	7.6±0.55
28 dpp	5.4 ± 0.55	5.8 ±0.45	5.4 ±0.55
35 dpp	5.4 ± 0.55	5.4 ± 0.55	5.8 ± 0.45
42 dpp	4.6 ± 0.55	5.0 ± 1.00	5.8 ± 0.45

*Days Post priming

higher peak at an earlier day after Ist injection.

Table 5 showed the result of the Memory Factor for various dilutions. The memory factors calculated for the experiment were used in assessing the efficacy of booster application and to evaluate the specific immune memory. The MF showed results more or less agreeing with that of the antibody response. The highest memory factor (0.88) was recorded in the *Mugil cephalus* injected with 10^{-7} CFU/ fish at 21 dpb.

Table 2: Anova for testing significance of difference between the doses and durations of priming response in *M. cephalus* to intraperitoneally injected *Vibrio vulnificus*

Summary	Count	Sum	Average	Variance
0ddp	3	2	0.66	0.01
7ddp	3	13.2	4.44	1.96
14ddp	3	17.2	5.73	2.17
21ddp	3	20.4	6.8	0.52
28ddp	3	16.6	5.53	0.05
35ddp	3	16.6	5.53	0.05
42ddp	3	15.4	5.13	0.37
10^6	7	29.8	4.25	3.6
10^7	7	33	4.71	3.76
10^8	7	38.6	5.51	5.06

Source of Variation	SS	df	MS	F	P-Value	F crit
Rows	69.88	6	11.64	30.2	1.434E-06	2.99
Columns	5.66	2	2.83	7.35	0.008	3.88
Error	4.62	12	0.38	-	-	-
Total	80.18	20	-	-	-	-

Treatment means are highly significant different from one another (P>0.05)

Table 3: Immunization of *Mugil cephalus* with formalin inactivated *Vibrio vulnificus* (booster dose)

Dpp*	Dilution					
	Log^{-6}		Log^{-7}		Log^{-8}	
0	0.6	0.55	0.8	0.45	1.0	0.70
7dpp	3.4	0.55	4.6	0.55	5.2	0.45
14 dpp	5.0	0.70	5.4	0.55	7.6	0.55
21dpb	6.2	0.45	6.8	0.45	7.6	0.55
28dpb	7.4	0.55	8.2	0.45	10.2	0.45
35 dpb	8.8	0.45	10.2	0.45	11.6	0.55
42 dpb	9.2	0.45	11.0	1.00	12.4	0.55

dpp - Days Post Priming

dpb - Days post booster

The vaccination trials for assessing the Humoral Immune Response using the agglutination test. Antibody titre test, revealed that after primary and secondary immunization with injection, immunized fish recorded the higher antibody titre. Injection of vaccine ensures that each fish receives a constant and exact dose of product. Alexander (1980) and Harris (1973) reported the intramuscular injection was more suitable for induction of primary response than methods giving the best protection,

Table 4: Anova for testing significance of difference between the doses and durations of priming response in *M. cephalus* to intraperitoneally injected *Vibrio vulnificus*

Summary	Count	Sum	Average	Variance
0 dpp	3	2	0.66	0.01
7dpp	3	13.2	4.4	0.84
14dpp	3	18	6	1.96
21dpb	3	20.6	6.86	0.49
28dpb	3	25.8	8.6	2.80
35dpb	3	30.6	10.2	1.96
42dpb	3	32.6	10.87	2.57
10^{-6}	7	40.6	5.8	9.45
10^{-7}	7	46.8	6.68	12.7
10^{-8}	7	55.6	7.94	15.7

Source of Variation	SS	df	MS	F	P-Value	F crit
Rows	223.63	6	37.273	121	4.961E-10	2.9961
Columns	16.23	2	8.116	26.4	4.0248E-05	3.8852
Error	3.687	12	0.307	-	-	-
Total	243.55	20	-	-	-	-

Treatment means are highly significant different from one another (P>0.05)

Table 5: Immune response and memory factor (mf) of immunised *Mugil cephalus* to different doses of formalin-inactivated *Vibrio vulnificus*

dpp/	10^{-6}		10^{-7}		10^{-8}		MF		
dbp	Primed	Booster	Primed	Booster	Primed	Booster	10^{-6}	10^{-7}	10^{-8}
0 dpp	0.6 ± 0.55	0.6 ±0.55	0.6 ± 0.55	0.8 ± 0.45	0.8 ± 0.45	1.0 ± 0.70	-	-	-
7 dpp	3.0 ± 0.70	3.4 ± 0.55	4.4 ± 0.55	4.6 ± 0.55	5.8 ±0.45	5.2 ± 0.45	-	-	-
14 dpp	4.6 ± 0.55	5.0 ± 0.70	5.2 ± 0.45	5.4 ± 0.55	7.4 ± 0.55	7.6 ± 0.55	-	-	-
21 dpb	6.2 ± 0.45	6.2 ± 0.45	6.6 ± 0.55	6.8 ± 0.45	7.6 ± 0.55	7.6 ± 0.55	-	-	-
7 dpb	5.4 ± 0.55	7.4 ± 0.55	5.8 ± 0.45	8.2 ± 0.45	5.4 ± 0.55	10.2 ± 0.45	0.22	0.276	0.481
14 dpb	5.4 ± 0.55	8.8 ± 0.44	5.4 ± 0.55	10.2 ± 0.45	5.8 ± 0.45	11.6 ± 0.55	0.48	0.066	0.69
21 dpb	4.6 ± 0.55	9.2 ± 0.45	5.0 ± 1.00	11.0 ± 1.00	5.8 ± 0.45	12.4 ± 0.55	0.65	0.88	0.828

dpp - Days Post Priming

dpb - Days post booster

more beneficial than immersion and bathing vaccination method.

Vaccinated fish appear to grow and survive better than their unvaccinated group. Moreover, the extensive use of antibiotic is undesirable because of development of resistant strains of bacteria and possible adverse effects on the aquatic environment. Hence the studies clearly showed the effective vaccine development will certainly help the aquaculture industry for producing the fishes which are free from diseases.

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