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Evaluation of the Efficiency of *Aeromonas hydrophila* Biofilm Vaccine in *Labeo rohita* Employing Monoclonal Antibody based ELISA

Vinay TN[1]*, Rajreddy Patil[2], Suresh Babu PP[2], Rajesh Rana[1] and Shankar KM[2]

[1]*Department of Fish Genetics and Biotechnology, central Institute of Fisheries Education, Mumbai, India*
[2]*Fish pathology and biotechnology laboratory, Department of Aquaculture, College of Fisheries, Mangalore, India*

Abstract

Specific detection of immunoglobulin (Ig) in fish serum is important in evaluation of vaccines. A monoclonal antibody based Enzyme Linked Immunosorbant Assay (ELISA) was developed to evaluate the immune response in rohu (*Labeo rohita*) to *Aeromonas hydrophila* biofilm vaccine. Anti rohu immunoglobulin MAbs were used to detect Immunoglobulin (Ig) of rohu in an indirect ELISA, specificity of which was determined by appropriate positive and negative controls. Fish were orally vaccinated with biofilm and free cell of *A. hydrophila* (10^{10} CFU/g fish/day) for 20 days and monitored for serum antibody production up to 60 days post vaccination. Serum from biofilm vaccinated fish recorded the highest antibody titer (0.40 ± 0.05, 0.52 ± 0.03, 0.50 ± 0.02, 0.62 ± 0.04, 0.51 ± 0.03, 0.61 ± 0.02 and 0.67 ± 0.009) followed by that from free cell vaccinated (0.12 ± 0.01, 0.20 ± 0.03, 0.13 ± 0.01, 0.21 ± 0.01, 0.10 ± 0.01, 0.20 ± 0.01 and 0.13 ± 0.01) and control fish (0.06 ± 0.006, 0.07 ± 0.002, 0.02 ± 0.008, 0.06 ± 0.01, 0.09 ± 0.004. 0.07 ± 0.005 and 0.049 ± 0.01) at 0, 10, 20, 30, 40, 50 and 60 days post vaccination respectively. Furthermore, sensitivity of ELISA was higher compared with agglutination antibody titer. The serum dilution used for ELISA was 1:1000, while the positive reaction in agglutination assay was observed at 1:256, 1:64 and 1:4 in biofilm vaccinated, free cell vaccinated and control respectively. The Relative percent survival (RPS) following challenge with *Aeromonas hydrophila* (10^6 CFU/ml) was found to be significantly higher in rohu fed with biofilm vaccine (83.4%) compared to that with free cell vaccine (25%).

Keywords: Biofilm vaccine; *Aeromonas hydrophila*; Enzyme Linked Immunosorbant Assay; *Labeo rohita*

Introduction

Culture of Indian major carps (IMC) contributes more than 80% of the total aquaculture production in India. *Labeo rohita* (rohu) with its high consumer preference and good growth rate, it is widely cultured in Indian subcontinent [1-3]. There has been a phenomenal shift from extensive to intensive culture of carps in the last three decades. Intensive aquaculture offers an increased opportunity for spreading of infectious diseases at all stages of production [4]. Among the bacterial pathogens *Aeromonas hydrophila* is a ubiquitous secondary pathogen of IMC including rohu. Several instances of infections with *A. hydrophila* in India have been reported in IMC in recent past [5] hence; vaccination of aquacultured fish is becoming inevitable with increasing health risks.

Since, the aim of vaccination is to increase the immune memory/humoral antibody response against the specific antigen, the detection of these specific antibodies is very essential to evaluate the efficacy of a vaccine [6]. The conventional serum agglutination assay though used often, has a major limitation of its lesser sensitivity and it is applicable when the antibody levels are relatively high [7]. The development of rapid and highly specific immunoassay has made it possible to study in detail of the immune response of fish. ELISA, in particular has been proven to be useful in detection of antibody titer in fish [8]. Furthermore, the availability of monoclonal antibodies, against fish IgM has made it still more effective and specific for precise evaluation.

This study was focused on evaluation of humoral response of *Labeo rohita* fingerlings by developing a monoclonal antibody (MAb) based indirect ELISA. Fish were orally vaccinated with biofilm vaccine and free cell vaccine of *A. hydrophila* which was previously demonstrated successfully by Azad et al. [9-12] and Nayak et al. [13]. The MAb used were produced in our laboratory against IgM of *Labeo rohita* [14]. The protection against the pathogen was evaluated by a homologous challenge with a virulent strain of *A. hydrophila*.

Materials and Methods

Source, isolation and maintenance of *Aeromonas hydrophila*

The bacterium was isolated from an infected *Labeo rohita* from a culture pond at college of fisheries, Mangalore. The colonies thus obtained were grown in tryptone soya broth (1.5% w/v) and harvested by centrifugation at 3000 g for 10 min and cell pellet was resuspended in sterile Tryptic Soya Broth (TSB) (Hi Media, India) and preserved by addition of 15% glycerol at -20°C. For use, the culture was revived on nutrient agar (NA) (2.8% w/v, Hi Media) slants and stored at 4°C until used.

Fish

Labeo rohita (rohu) were acclimatized in the laboratory in fiberglass tanks (1000 L) filled with fresh water (26°C) and continuous aeration. During this period, fish were fed with control diets at 2% of body weight. Water was replenished to the extent of 40-50% on alternate day.

Formulation of biofilm and free cell vaccine diet

Biofilm vaccine and free cell vaccine of *Aeromonas hydrophila* was prepared according to the method described by previous authors

***Corresponding author:** Vinay TN, Department of Fish Genetics and Biotechnology, central Institute of Fisheries Education, Mumbai, India, Tel: +82-10-2947-1983; E-mail: vinaytn56@gmail.com

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[9]. Briefly, *A. hydrophila* isolate was grown on chitin flakes (0.3%) suspended in TSB (0.255% w/v) and incubated at 37°C for 4 days with 6 h agitation at 180 rpm on a rotor shaker every day. The biofilm cells were harvested along with chitin and heat inactivated at 100°C for 50 min. For free cells, 1 day old culture grown in TSB (1.5% w/v) was harvested and heat inactivated at 90°C for 10 min.

Biofilm and free cell vaccine diets were prepared by mixing heat inactivated biofilm (10^{10} cfu/g) or free cells (10^{10} cfu/g) with cooked and cooled feed ingredients, palletized and sun dried. A control diet without antigen was also prepared.

Experimental design

Labeo rohita fingerlings (15.2 ± 1.4 g) were randomly selected from the stock and transferred to individual plastic tubs in three groups, each treatment consisted duplicates with 30 fishes, group A and B were fed with biofilm and free cell incorporated feed respectively for 20 days, fish in group C was fed with control diet. After 20 days of oral immunization, 5 fish from each group were bled through the caudal vein at 0, 10, 20, 30, 40, 50 and 60 days post immunization (dpi). Blood was collected from the caudal vein of five fish from each of the biofilm, free cell vaccinated and control groups, kept at room temperature for 1 hour and at 4°C overnight, and then centrifuged at 737 × g for 10 min at 4°C to separate serum. Serum was stored at -20°C until use.

Enzyme-linked immunosorbant assay (ELISA)

The ELISA was carried out according to Furuta et al. [15] with relevant modifications. Briefly, antigen was coated to ELISA plate at 1 µg/well using carbonate-bicarbonate buffer, pH 9.6 and incubated overnight at 4˚C. Unbound antigen was poured off and the plate was washed thrice with wash buffer (0.05% Tween 20 in PBS) and once with PBS. Free sites on well were blocked by incubating the wells with 300 µl blocking solution (5% Skimmed milk powder in PBS) for 2 h at room temperature. Plate was washed three times with wash buffer (PBS-Tween 20) and fish sera from biofilm/ free cell/ control fish at different DPI at 1:1000 dilutions was added at the rate of 100 µl/ well in duplicates and incubated at room temperature for 2 h. After washing, 100 µl/well of anti-rohu IgM monoclonal antibody (F2D9) was added and incubated for 2 h. Washing again, rabbit- anti mouse IgG peroxidase (Bangalore Genei) at 1:2000 dilutions, diluted with 3% BSA-PBS was added at 100 µl/well followed by 1h incubation. Washing thrice with PBST and twice with PBS, 100 µl/well of substrate (TMB-H_2O_2 Bangalore Genei) diluted with distilled water 1:20, was added and incubated for 5-10 min. The reaction was stopped by adding 50 µl 1M H_2SO_4. Optical density (OD) was read at 450 nm using ELISA reader (Biotek). Background (OD) was assessed in wells with no antigen, and wells with no fish serum.

Agglutinating antibody titer

Assay was performed as described by earlier author [13]. Briefly, 90 µl of immune/control serum (60 dpv) was serially diluted with 50 µl PBS pH 7.2 and the 50 µl (10^9 cfu/ml) of heat inactivated free cell suspension of *A. hydrophila* was added. Plates were incubated at room temperature overnight prior to microscopic examination (X40) for agglutination. Results were expressed as positive or negative for agglutination.

Challenge studies

On 60 day post vaccination (dpv), fish were moved to a challenge facility, 1 day before challenge and distributed into aquaria containing fresh water. Fifteen fish from each treatment group and control group were challenged intra peritoneally with 10^6 cfu/ml of viable *A.hydrophila* to assess the overall protection. A naive group was maintained and observed for 18 days post challenge (dpc) for appearance of gross clinical signs and mortality pattern post challenge. Blood and kidney samples were taken aseptically from dead and moribund fish, plated on nutrient agar and *Aeromonas* selective medium (Hi Media) to confirm specific cause of death and morbidity.

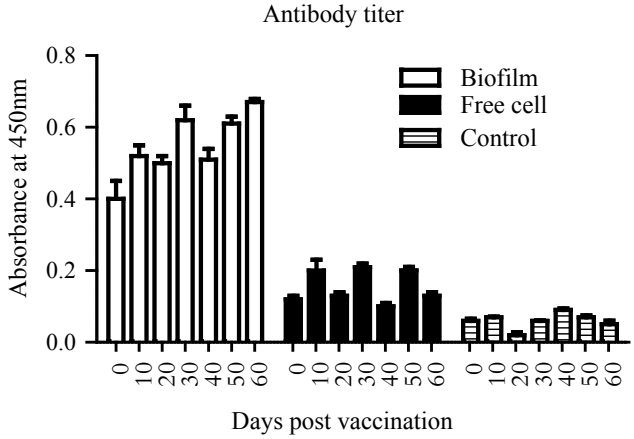

Figure 1: Antibody titer of *Labeo rohita* following oral vaccination with biofilm and free cell of *Aeromonas* hydrophila.

Vaccine	Serum dilution									
	0	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
Biofilm	+	+	+	+	+	+	+	+	+	-
Free cell	+	+	+	+	+	+	+	-	-	-
Control	+	+	+	-	-	-	-	-	-	-

(+); positive agglutination, (-); No agglutination.

Table 1: Summary of agglutinating antibody titer.

Statistics and relative percent survival (RPS)

Kaplan–Meier curves and log rank test were carried out using GraphPad Prism5 Software for survival analysis of all challenge trials. RPS was calculated according to the method described by Amend [16].

RPS = [1-(% mortality of vaccinated group/ % mortality in control)] × 100

Results

Antibody response

A monoclonal antibody based ELISA was employed to evaluate the humoral response of the biofilm vaccinated and free cell vaccinated fish. Serum from five fish was used and 1:1000 diluted serum was used to evaluate the antibody response. Antibody response of biofilm vaccinated fish was significantly enhanced compared to free cell and control group. Figure 1 shows the average antibody titer obtained in biofilm, free cell and control diet fed fish.

Agglutinating antibody titer

Serum subjected to antibody agglutination assay showed positive reaction up to 1:256 dilutions in biofilm vaccinated group and 1:64 dilutions in free cell vaccinated group and it was detected at 1:4 dilutions. The dilutions and the positive reaction for agglutination are given in the Table 1.

Protection upon challenge

The log-rank test showed a significant difference in survival percentage between the biofilm vaccinated and free cell vaccinated fish with *p*-values of 0.0073 and the significance between biofilm vaccinated and non-vaccinated control was still high with *p*-values of 0.0002. There was no significant difference in the survival percentage of free cell vaccinated and non-vaccinated control group. Figure 2 shows the percentage survival of biofilm and free cell vaccinated fish.

Relative percent survival (RPS) of biofilm and free cell with homologous challenge with *A.hydrophila* (10^6 CFU/ml) are given below in Table 2. The RPS for free cell and biofilm were determined as 25 and 83.4% respectively. The protection level was found to be dependent on type of vaccine.

Discussion

In this present study we employed a MAb based ELISA to detect specific serum antibodies, induced by *A.hydrophila* biofilm vaccine and free cell vaccine which was delivered orally for a period of 20 days and got encouraging results, showing the same trends of protective response to the challenged pathogen, as previous studies [9-13].

The accurate detection of specific antibodies against the antigen is essential for the effective evaluation of vaccines [6,17]. Immunoglobulin in fish sera can be detected by several techniques such as, immunodot, western blot, agglutination titers and ELISA, among which ELISA and agglutination titer are mostly applied as quantitative assays where ELISA is more sensitive [18]. The results show that the antibody titer magnitude measured here was significantly higher in biofilm vaccine than free cell vaccine. The role of biofilm in generating serum antibody response was found to be greater than free cell vaccine in this study in agreement with the previous studies of Azad et al. [9-12] and Nayak et al. [13]. However, those studies failed to detect the specific antibodies against the antigen as they employed agglutination assay for measuring the antibody titer. ELISA can be employed as a potential tool to detect specific antibodies in fish post vaccination [19]. The present results, provides more strength to the previous findings and encourages the use of biofilm of microbes for efficient oral vaccine.

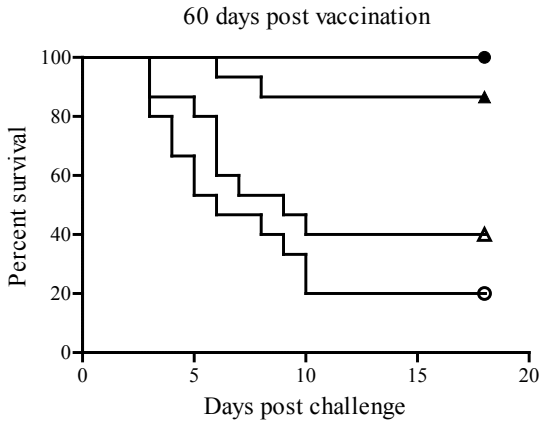

Figure 2: Kaplan–Meier curve showing survival of *Labeo rohita* challenged at 6 days post vaccination. Naïve group (●), biofilm vaccinated (▲) free cell vaccinated (Δ) and control (○) group following an intraperitoneal injection with *Aeromonas hydrophila* (10^6 CFU/ml). A significant difference was noted between the biofilm vaccinated and free cell vaccinated groups with a *p*-value 0.0073.

Group	Challenge dose	Challenge temp.	No. of Fish	Percentage mortality	RPS (%)
Biofilm	10^6 CFU/ml	28°C	2/15	13.3*	83.4
Free cell	10^6 CFU/ml	28°C	9/15	60	25
Control	10^6 CFU/ml	28°C	12/15	80	
Naive	NA	28°C	0/15	0	100

NA; Not applicable, *; significant survival percentage than free cell vaccine (*p*=0.0073)

Table 2: Summary of challenge studies.

The sensitivity of ELISA is observed to be much higher compared to the agglutination titers. The ELISA was able to detect specific antibodies at higher serum dilutions at 1:1000 as compared with lower detection limits of the agglutination assay, which could show the positive reaction at 1:256 and 1:64 in biofilm and free cell vaccinated fish serum respectively. The results are in agreement with the previous studies of Furuta et al. [15] where they reported the sensitivity of ELISA was six times higher in detecting flounder immunoglobulins than agglutination assay and Yoshimizu et al. [20] reported the sensitivity of ELISA to be several times higher than agglutination assay.

We have shown that the fish vaccinated orally with biofilm of A.hydrophila, showed elevated antibody titer as well as a good protection against the virulent *A.hydrophila* challenged via intra peritoneal injection. The increased antibody titer and protection against challenged bacteria in biofilm vaccinated fish is due to the property of biofilm, not being destroyed by the digestive enzymes and being available in large quantity to the lymphoid organs of fish to develop adaptive immune response against the antigen, which is also evident in previous studies [12,13]. Furthermore, the failure to produce enough specific antibodies in free cell vaccinated group is due to the destruction of the antigen by digestive enzymes before reaching the hind gut [12,13]. The immune stimulatory role of chitin, added along with the biofilm vaccine may not be denied, as chitin can enhance the innate immune response of the fish [21] which ultimately might have led to the adaptive response to produce increased antibody titer. But, the role of chitin in enhancing the immune response along with biofilm needs a detailed study.

With the above results, we conclude that the biofilm oral vaccine can be efficiently employed in the culture system to overcome the infectious diseases and ELISA is much more sensitive in detecting specific serum antibodies in vaccinated fish serum. So, it can be used as a tool to evaluate the efficacy of vaccines.

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