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# Evaluation of biofilm of Vibrio anguillarum for oral vaccination of Asian seabass, Lates calcarifer (BLOCH, 1790)

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ABSTRACT

The present study evaluated the biofilm (BF) of *Vibrio anguillarum* for oral vaccination of Asian seabass, *Lates calcarifer*. An 80-day experiment was carried out in circular fiber-reinforced plastic (FRP) tanks using free cell (FC) and BF of *Vibrio anguillarum* with triplicate in each. Heat-inactivated FC and BF cells at $10^7$, $10^{10}$ and $10^{13}$ CFU/g fish/d were fed to fish for 20 days, agglutination antibody titer estimated at each 10 days interval up to 60-day post vaccination. As compared to FC and control there was a significant increase in agglutinating antibody titer in the biofilm vaccinated fishes. Among the 3 doses, BF at $10^{10}$ cfu/g fish/d was considered the ideal dose for vaccination. Relative percentage survival (RPS) was higher in biofilm vaccinated fish (85.4%) compared to that with free cells (27.0%). The study demonstrated the better performance of *V. anguillarum* biofilm oral vaccine compared that with free cell vaccine in *L. calcarifer*. The study further supports better performance of biofilm vaccine model with one more bacterial pathogen in a high carnivore fish.

## 1. Introduction

Asian seabass, *Lates calcarifer* is one of the important culture species in South East Asian countries for its taste and meat texture. Due to intensification and rapid expansion of Asian seabass culture, there is a prevalence of a number of diseases, especially those caused by *Vibrio spp*., which is of economic importance. Vibriosis characterized by hemorrhagic septicemia is a major disease occurring in marine and brackish water fish such as Asian seabass, salmon, cod, halibut, Japanese eel, rainbow trout, and ayu, as well as shellfish such as shrimps. Vibrios, are members of the genus *Vibrionaceae* are gram-negative, motile, rod-shaped mesophilic and chemoorganotrophic bacteria [1]. *Vibrio harveyi* and *Vibrio anguillarum* are most frequently isolated marine *Vibrio* species [2], associated with large-scale loss of larval and juvenile fishes [3]. *Vibrio anguillarum* is the causative agent of vibriosis Asian seabass and is the major obstacle for its culture practice.

Efforts were made to overcome the disease caused by *Vibrio anguillarum* by using antibiotics and vaccines. In Asia, with the exception of Japan, vaccines are not commonly used for fish disease control [4]. Several attempts were made for prevention of the disease outbreak caused by *V. anguillarum* using free cell (FC) vaccine but with no significant outcome [5]. Currently, there are a number of live attenuated, inactivated/killed, toxoid (inactivated toxin) vaccines commercially available for use in fish [6]. These can be administered through different routes, among them, oral administration is best for aquaculture because it is free of stress, cheap, easy for mass administration. But, it is least effective in terms of antibody titer and protection due to vaccine antigen degradation in the stomach and anterior intestine of fish [7].

Earlier studies conducted in our laboratory on the evaluation of biofilm of *Aeromonas hydrophila* for oral vaccination of freshwater fish such as carp -a herbivore [8], *Clarias batrachus* an omnivore [9] and *Channa striatus* a carnivore [10], have been successful with a significant increase in antibody titer and protection upon challenge. Biofilm vaccine model got further strengthened with a demonstration of localization and distribution of antigen in larger quantities for longer duration in the gut and lymphoid tissues following oral vaccination [11]. Against this background, the present study was aimed to develop and evaluate biofilm of *V. anguillarum* for oral vaccination in Asian seabass a highly carnivorous with the quite different digestive environment and enzyme activity like pancreatic and intestinal enzymes [12].

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## 2. Materials and methods

### 2.1. Bacterial isolate and its maintenance

*Vibrio anguillarum* isolate was obtained from the Cochin University of Science and Technology (Dr. I.S. Bright Singh) and reconfirmed by PCR using species-specific primer, according to Gyeong et al. (2007) [13]. The confirmed isolate was grown in 1.5% (w/v) Tryptic Soya Broth (TSB) supplemented with 2% NaCl and harvested by centrifugation at 10,000 rpm for 10 min. The cell pellet was re-suspended in sterile TSB (1.5% w/v + 2% NaCl) with 15% glycerol and aliquoted into 1.5 ml microcentrifuge tubes for storing at −80 °C for further use.

### 2.2. Fish stock maintenance

Asian seabass, *Lates calcarifer* advance fingerlings acclimatized to 10 ppt of salinity were procured from Rajiv Gandhi Centre for Aquaculture (RGCA), Nagapattinam, Tamilnadu. The fishes were acclimatized in the fish farm of College of Fisheries, Mangalore. During acclimatization, fish were provided with artificial feed.

### 2.3. Preparation of Vibrio anguillarum biofilm cells

*Vibrio anguillarum* biofilm was prepared according to Satish (2013) [14]. Briefly, the conical flask containing TSB (0.1% w/v, Himedia, Mumbai) supplemented with 2% NaCl and chitin flakes (0.3% w/v, Sigma) was autoclaved at 121 °C for 15 min and cooled to room temperature. The medium was inoculated with one ml of *Vibrio anguillarum* culture in log phase (18 h) and agitated for 6 h daily in an incubator mechanical shaker (120 strokes/min) set at room temperature. On the third day, the supernatant was decanted and the substrate (chitin flakes) was washed thrice in the same flask with sterile phosphate buffer saline (PBS, pH 7.2) to remove the free cells. The chitin flakes were transferred to a centrifuge tube with 10 ml PBS and agitated vigorously for 4 min to dislodge the biofilm cells from chitin flakes. Biofilm containing supernatant was aseptically transferred to another centrifuge tube and biofilm cells harvested by centrifugation. The cells were further washed thrice using sterile PBS (pH 7.2) and finally, the pellets were re-suspended in PBS. Biofilm cells were then heat inactivated at 80 °C for 60 min before incorporating in the feed.

### 2.4. Preparation of Vibrio anguillarum free cells

*Vibrio anguillarum* free cell was prepared according to Satish (2013) [14]. Briefly, TSB (1.5% w/v) with 2% NaCl was sterilized at 121 °C for 15 min and cooled to room temperature. The medium was inoculated with 24 h old *Vibrio anguillarum* culture and the flasks incubated at room temperature for 24 h. The cells were harvested by centrifugation at 10,000 rpm for 10 min and further washed thrice using sterile PBS (pH 7.2) and finally, the pellets were re-suspended. The free cells were heat inactivated at 60 °C for 10 min before incorporating in the feed.

### 2.5. Incorporation of biofilm and free cell oral vaccine in feed

Biofilm and free cells were incorporated in the feed according to Prabhu et al. (2014) [10]. Briefly, Feed ingredients such as fish meal (55%), rice bran (18%), groundnut oil cake (10%), tapioca flour (10%) were mixed together, cooked and cooled to room temperature. cod liver oil – 5% (v/w) and vitamins – minerals mixture – 2% were mixed with cooked ingredients, followed by incorporation of heat-inactivated biofilm cells (BF) and free cell (FC). The feed paste was pelletized and sun-dried and dry pellets were stored at 4 °C in the refrigerator. A control diet (C) was prepared with sterile PBS (pH 7.2) instead of biofilm or free cells of *V. anguillarum*.

**Table 1**
Details of oral vaccination of Asian seabass with *Vibrio anguillarum*.

| Vaccine type | Vaccine dose cfu/g f/ day | No. fish per tank | Weight of fish (g) |
|---|---|---|---|
| Biofilm vaccine | $10^7$, $10^{10}$, $10^{13}$ | 60 | 8 ± 0.50 g |
| Free cell | $10^7$, $10^{10}$, $10^{13}$ | 60 | 8 ± 0.50 g |
| Control | 0 | 60 | 8 ± 0.50 g |

### 2.6. Oral vaccination of Asian seabass with V. anguillarum

FRP tanks (250 L) were set up for the treatment Biofilm (BF), free cell (FC) and Control (C), and each tank was stocked with 60 fingerlings (8 ± 0.50 g) (Table 1). Three doses of biofilm and free cells ($10^7$, $10^{10}$ and $10^{13}$ cfu/g fish/day respectively) in triplicates were used. The fish were acclimatized in brackish water (10 ppt) with a control feed (C) for a week followed by starvation for 24 h. Fish were fed with biofilm (BF) and free cells (FC) incorporated feed at $10^7$, $10^{10}$ and $10^{13}$ colony forming units (CFU) per gram fish per day (cfu $g^{-1}$fish $d^{-1}$) respectively for 20 days. The control was fed with a control feed (C). Complete acceptance of feed was insured each day. Vaccine incorporated feed was withdrawn after 20 days and fish were fed with normal feed at 3% of body weight per day till the end of the experiment. Water in the tanks was replenished to an extent of 50% on alternate days.

### 2.7. Collection of serum for estimation of antibody titer

After feeding vaccine for 20 days, five fish from each dose and control were bled from their caudal veins with non-heparinized disposable syringes on 0, 10, 20, 30, 40, 50 and 60-day post vaccination (dpv). Blood was collected in microcentrifuge tubes and allowed to clot at room temperature for 1 h, followed by overnight incubation in refrigerated condition (4 °C). Supernatant clot free serum was collected following centrifugation at 10,000 rpm for 10 min and inactivated at 50 °C for 30 min. Sera from 5 fish from each treatment were pooled and stored in aliquots at −20 °C.

### 2.8. Agglutination titers

Agglutination titers of serum were determined with heat-inactivated cells of *Vibrio anguillarum* according to Azad et al. (1999) [8]. Sterile PBS (pH 7.2) was added (50 µl) to each well of a 96 well 'U' bottom microtiter plate. The first plate was added with 100 µl of inactivated serum. From the first well 50 µl of inactivated serum was transferred to the second, mixed well. From the second well, 50 µl was transferred to third and this serial double dilution was continued till the 11th well. The serial dilutions so obtained, were 0 in the first well, 1:1 in the second well, 1:2 in the third and so on till 1:512 in the 11th well. The last well without serum in each row served as negative control. Later, heat inactivated *V. anguillarum* suspension (OD = 0.9 at 575 nm) was added to each well (50 µl) and mixed. The plates were incubated at room temperature for 1 h and overnight at 4 °C. Agglutination titers for each fish samples were expressed as $\log_2$ values based on visual observations [15].

## 3. Protection upon challenge

### 3.1. Determination of $LD_{50}$ of V. anguillarum

*L. calcarifer* (8 ± 0.50 g) were maintained in nine plastic tubs (80 l capacity), in triplicate, with 10 fish in each tub. Prior to the experiment, fish were randomly checked for the presence of *V. anguillarum* in blood. *V. anguillarum* maintained on nutrient agar slant was cultured in 1.5% TSB with 2% NaCl for 18 h on a shaker. The cells were harvested, re-suspended in PBS and serially diluted with sterile normal saline for

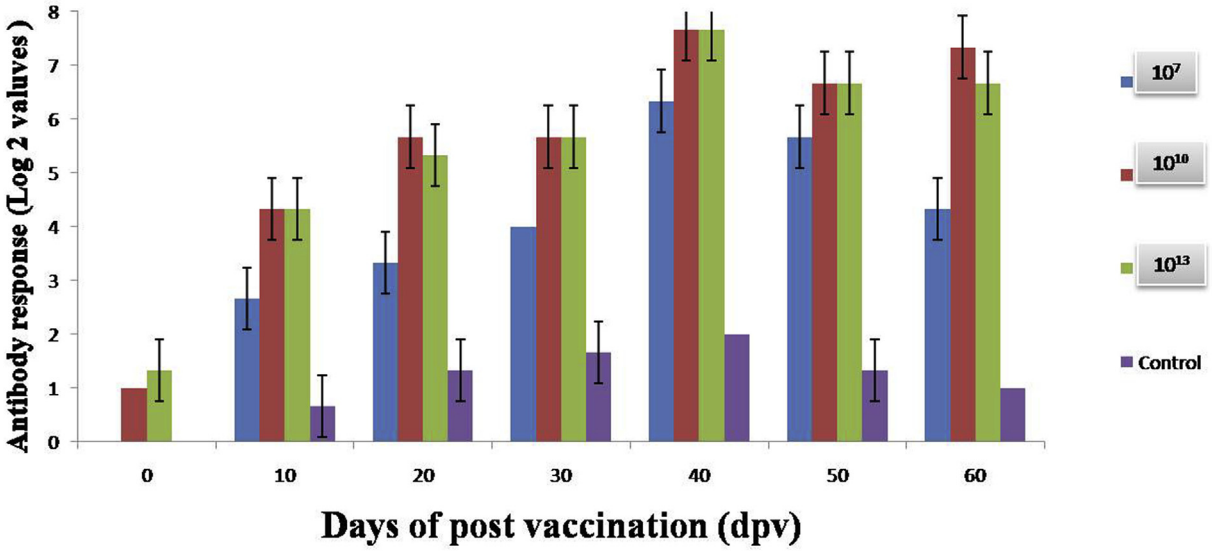


**Fig. 1.** Antibody response in Asian seabass with the varying dose of BF cells.

enumeration on TSA plates. Fish in eight tubs were injected (IM) each with $10^4$, $10^5$, $10^6$, $10^7$, $10^8$, $10^9$ and $10^{10}$ CFU/g fish in 0.5 ml normal saline respectively. Control fish in tub 9 received 0.5 ml of normal saline only. Mortality was recorded at the end of 6, 12, 24, 48, 72, 96, 120, 144, 168, 192, 216 and 240 h post injection. Kidney of moribund fish was used for re-isolation of the bacterium using selective TCBS agar. The degree of virulence ($LD_{50}$) was calculated according to Probit Analysis (PA) method. The dose $10^6$ CFU/g fish was selected and used for challenge studies.

### 3.2. Challenge with V. anguillarum

At the end of 60-days post vaccination (dpv), antibody titer was determined by microtiter method (agglutination) and there was a significant difference in the antibody titer of experimental animal's blood serum at different dose of treatment. Among the vaccinated group, $10^{10}$ cfu/gm fish/day treatment group was chosen for challenge study. Twenty orally immunized *L. calcarifer* from each BF, FC, and control group were selected and with the three replication on the 60th-day post vaccination (dpv) for the challenge. Each fish was injected (IM) with 0.5 ml of 18 h old culture of *V. anguillarum* at $10^6$ CFU/g fish. The appearance of gross clinical signs and mortality if any, were observed for 10 d. Blood and kidney samples were aseptically collected from the freshly dead and moribund specimens, homogenized and plated on nutrient agar and *V. anguillarum selective* TCBS agar (Himedia, Mumbai) and confirmed by PCR. Relative per cent survival (RPS) was calculated according to Amend (1981) as follows;

$$RPS = [1\text{-}(\%\ \text{mortality of vaccinated group}/\ \%\ \text{mortality in control}] \times 100$$

For statistical analysis, two way ANOVA, Duncan multiple range tests (DMRT) and Chi-square test were applied to compare the treatment means and dependence of survivability on vaccines respectively.

## 4. Results and discussion

In aquaculture, oral vaccination is the most preferred method to economically immunize fish of all size and age groups. However, poor response to oral vaccines [16], possibly due to the destruction of antigenic epitopes due to gut enzymes [17], poses a major hindrance for developing an effective oral vaccine. Furthermore, digestive enzyme activity varies with the size, age and feeding habit of the fish. In order to overcome destruction, vaccine delivery through encapsulated antigen microspheres [18], enteric coated vaccine [19], bioencapsulation vaccine in live feed [20] and nanoparticle [21] were developed but were found to be complex, costly and not practical. And the performances of these modified oral vaccines varied with herbivore and carnivore fishes. Our laboratory developed a novel biofilm vaccine against *A. hydrophila* infection in freshwater fishes with significantly higher antibody titer and protection upon challenge in herbivore, omnivore and carnivore models [9,10,22]. Later, we have also evaluated biofilm of *V. alginolyticus* in tiger shrimp (*Penaeus monodon*) wherein BF was found to be superior to FC in stimulating the non-specific immune response in the shrimp [23].

Among Vibrios, *V. anguillarum* causes major economic loss in the Asian seabass culture. Moreover, Asian seabass highly carnivorous in nature and reported to contain 100% animal pray in the stomach [24,25] and digestive enzyme activity is quite different from other carnivore fishes (Srichanum et al., 2013). Also, increased pinocytotic activity in 6-day-old and 14-day-old larvae and all digestive activity after 18-h post hatchlings of *L. calcarifer* has been reported by Walford and Lam (1993) [26] and Srichanun et al. (2013) [12]respectively. In this study, we have successfully evaluated the biofilm of *V anguillarum* in the highly carnivore seabass.

### 4.1. Immune response to a biofilm of V. anguillarum

Fishes were vaccinated for 20 days and the antibody response was monitored from the 21st day ($0^{th}$dpv) to 80th day at 10 days interval. Agglutination antibody response in Asian seabass to BF and FC oral vaccine is presented in Figs. 1–3. On $0^{th}$dpv, the antibody titer with BF and FC was similar to that of control groups. A progressive improvement in serum antibody titer and protective response with time following biofilm oral vaccination was observed. This is probably due to the enhanced uptake and longer retention of the biofilm antigens compared to that of free cell vaccine [22]. The possibility of biofilm antigen being available at the immune responsive hindgut with minimally altered immunogenic epitopes could have contributed to the observed higher titer and subsequent protection [17]. The glycocalyx of biofilm is a polymer of neutral hexoses [27] which encapsulates and possibly protects the bacterial surface antigens from digestion in the gut.

The antibody titer with FC was low compared to that with BF probably due to the destruction of vaccine molecule in the gut of the *L. calcarifer*. Similar results were observed in previous studies in freshwater fishes such as murrel [10], catfish [9] and carps [8,22].

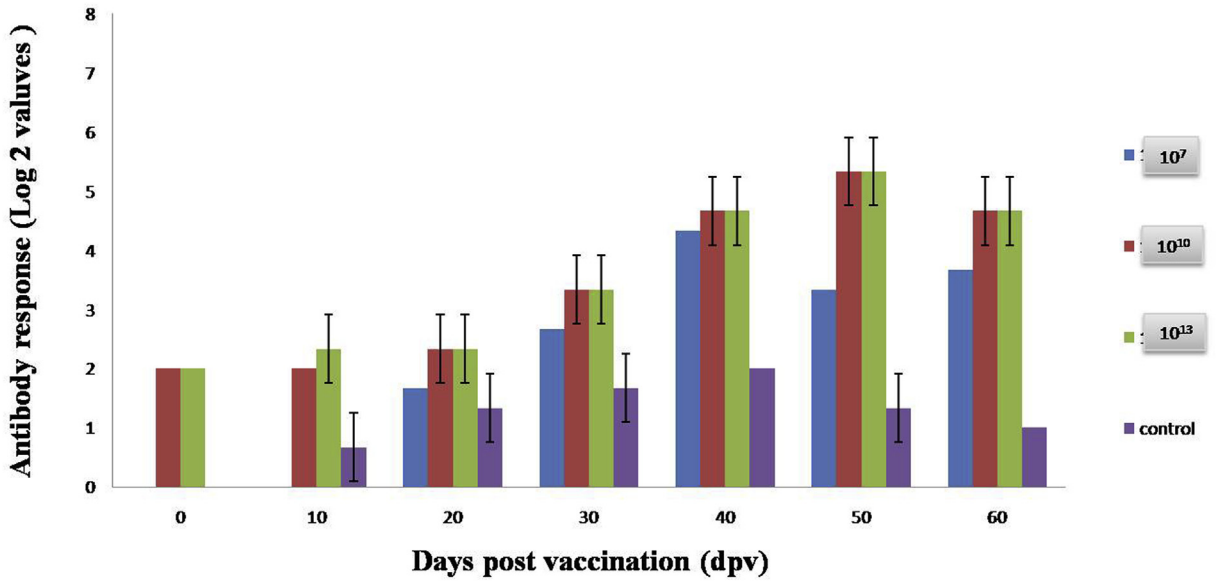


**Fig. 2.** Antibody response in Asian sea bass with the varying dose of free cells.

Therefore, BF vaccine has more relevance for oral vaccination of fishes. However, a difference exists in degradation, uptake, and processing of antigen in carps, catfish, and murrel as indirectly indicated by the antibody response.

The *V. anguillarum* biofilm vaccine elicited a dose-dependent antibody response with the highest titer and protection (RPS) at $10^{13}$ followed by $10^{10}$ & $10^{7}$ CFU/g fish/d. High dose priming is normally believed to elicit greater humoral response and memory in freshwater carp [28] and this appears to be true also in the case of orally administered biofilm vaccine. But, between the doses $10^{10}$ and $10^{13}$ cfu/g fish/d there was were having no significant difference in agglutination titer. Similar observations were made by Azad et al., (1997) [22] for BF *A. hydrophila* in freshwater carp. Hence $10^{10}$ cfu/g fish/d from each treatment group was chosen for challenge study.

### 4.2. Protection upon challenge with biofilm vaccine of V. anguillarum

$LD_{50}$ of *V. anguillarum* was determined by probit analysis method and found to be $10^{6}$ cfu/g fish (Fig. 4). Vaccinated Asian seabass showed elevated protection to the homologous injection challenge. In control, group survival was only 20% compared to 88.3% with biofilm and 41.6% with free cell vaccine. The relative percent survival (RPS) was 85.4 and 27.0 in biofilm and free cell vaccine respectively (Table 2). Similar studies with *Aeromonas hydrophila* biofilm oral vaccination in freshwater fishes such as murrel (*Channa straitus*), catfish (*Clarias batrachus*) and carps by Prabhu et al. (2014) [10], Nayak et al., (2004) [9]and Azad et al., (1997) [22] respectively demonstrated higher RPS in biofilm vaccinated compared to that with free cell vaccinated fishes upon homologous challenge. Finally, the study demonstrated the better performance of *V anguillarum* biofilm oral vaccine compared to that with free cell vaccine in brackish water fish *L. calcarifer*. The study further demonstrates the better performance of the biofilm oral vaccine model with one more bacterial pathogen in a highly carnivore fishes used in aquaculture.

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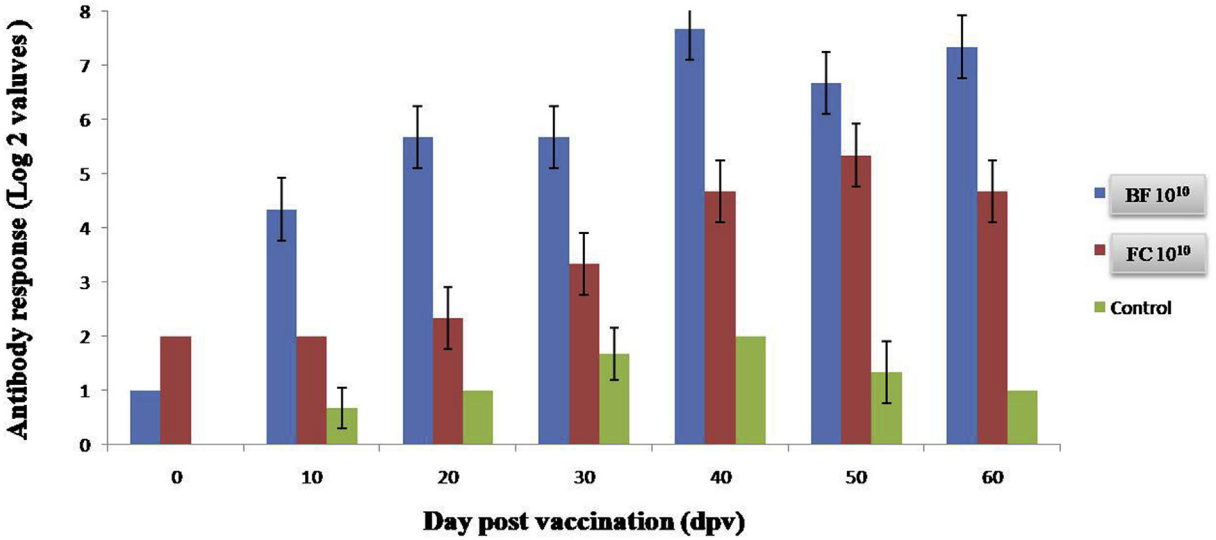


**Fig. 3.** Antibody response in Asian seabass with $10^{10}$ cfu/g f/d of FC, BF,and control (Statistically found a significant difference in immune response among the vaccines).

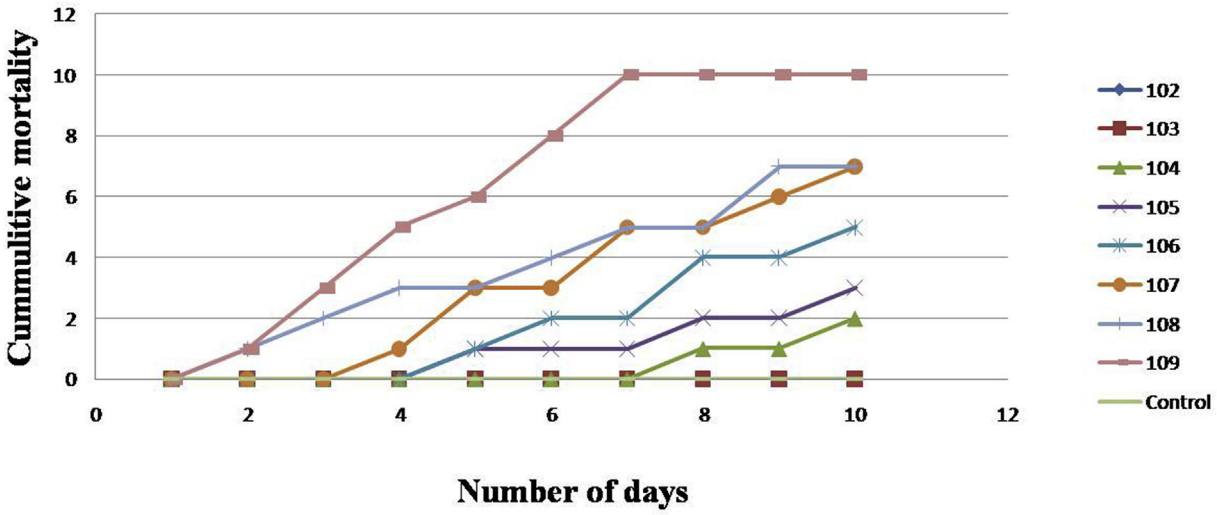


**Fig. 4.** $LD_{50}$ of *Vibrio anguillarum* to Asian seabass.

**Table 2**
Protection upon challenge with *Vibrio anguillarum* at $10^6$ cfu/g f.

| Treatments | No. of fish challenged | No. of fish survived | Mortality | % survival | RPS |
|---|---|---|---|---|---|
| Biofilm vaccine | 60 | 53 | 7 | 88.3 | 85.4 |
| Free cell vaccine | 60 | 25 | 35 | 41.6 | 27.0 |
| Control | 60 | 12 | 48 | 20 | - |

Biofilm vaccine survival is significantly different from that free cells cavvibe ($Z > 1.96$).

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.09.053.

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