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Infection and immunization trials of Asian seabass (*Lates calcarifer*) against fish pathogen *Vibrio anguillarum*

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Abstract: *Vibriosis is one of the most prevalent fish diseases caused by bacteria belonging to the genus Vibrio sp. Fish disease will be controlled by proper vaccination trials and maintenance of fish form. Pathogenicity for Asian seabass (Lates calcarifer) against V. anguillarum results in necrosis and haemorrhagic areas near the base of fins, exopthalmia and ulcers on the skin surface. Around 50, 100, 200 µl of formalin killed bacterial cells were injected (Intraperitoneal) to three different size (5-10, 20-30, 35-50g) of seabass fishes respectively and control sere as saline were maintained separately. The Relative Percentage Survival (RPS) for vaccinated fishes was 60, 75, and 62.5 respectively and the vaccinations for 20-30 g fishes stay alive. These results stated that the vaccination for fishes with 20-30 g size may fabricate good immune response.*

Key words: *Seabass, Vibrio, Antigen, Immunization, Fish*
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Introduction

Aquaculture industry is a boon to mankind that helps to meet the growing food demand. Bacterial disease outbreaks impose a significant constraint in fish and shellfish production. Vibriosis is one of the most prevalent fish diseases caused by bacteria belonging to the genus *Vibrio*. *Vibrio alginolyticus, V. parahaemolyticus, V. cholera* (non-O1), *V. vulnificus* (Biotype 2), *V. anguillarum, V. ordalii, V. damsela, V. carchariae* and *V.salmonicida* are considered to be predominant fish pathogens (Kim and Bang, 2008; Kannapiran *et al.,* 2009). In India, previous reports stated that *V. anguillarum* is the major causative agent in fish culturing ponds (Kumar *et al*., 2007). Previously, treatment of diseases has focused on chemicals and antibiotics. Treatment of affected fish with antibiotics is effective, but gives rise to problems such as accumulated resistance in the bacteria, which renders the antibiotic useless (Choi and Oh, 2007). However the increasing economical and social concern to decrease the use of antibiotics like chloramphenicol, nitrofurazone, oxolinic acid, oxytetracycline and sulphamerazine (Austin and Austin, 1993) other therapeutic chemicals used in fish farming has encouraged more environmentally friendly approaches to disease control (Verschuere *et al*., 2000). Moreover Li *et al*. (1999) stated that about 51 *Vibrio* isolates from diseased silver sea bream were resistant to these drugs. Kumar *et al*. (2007) developed the DNA vaccine against *V. anguillarum* causing acute vibrio hemorrhagic septicemia was evaluated in Asian seabass (*Lates calcarifer* Bloch), a common species of the Indian coast and a potential resource for the aquaculture industry. This approach may be tedious to control different bacterial pathogens. In this context, we scrutinized the fish disease and pathogenicity of *V. anguillarum* and its control over Asian seabass (*Lates calcarifer*) through formalin killed bacterial culture as vaccine.

Materials and Methods

Asian seabass (*Lates calcarifer*) of various weight (5-10, 20- 30, 35-50g) were collected from seabass hatchery (Rajiv Gandhi Centre for Aquaculture), Sirkazhi, Tamil Nadu, India and maintenance of Asian seabass (*Lates calcarifer)* fish were done in experimental tanks. The plant consists of 10 cylindrical 100 l insulated tanks respectively and three vertical 200 l insulated tank with UV treated seawater (20-35 ppt) filtered by 25, 10 and 1mm cartridge filters respectively. Internal aerator, with flow rate of 0.25 l min^{-1} for all tanks were used. Parameters like oxygen, temperature and salinity monitored twice a day. Fish were fed twice a day with commercial feed.

V. anguillarum was used in experimental infection on Asian seabass to confirm the pathogenicity and LD_{50} dosage. Bacterium *V. anguillarum* was cultured in the laboratory in Luria Bertani broth (Himedia, Mumbai) at 37°C for 24 hr and then the cells were separated by centrifugation at 8,000 × g and suspended in Phosphate buffer saline (PBS pH 7.0). Through serial dilution and plating method, viable cells were counted and different concentration of cells (10^4, 10^5, 10^6, 10^7 and 10^8 cells ml^{-1}) were injected intraperitoneally to fish with weight range of 50-60 g (Saeed and Plumb, 1986). The infected fish were collected, immobilized and spleens were dissected and fixed in phosphate buffered formalin. Paraffin thin sections at 6 µm were stained by the standard hematoxylin and eosin (Deivasigamani, 2007) and cell dimensions were measured by light microscope.

Antigens for vaccination were made using *V. anguillarum*, which were grown on nutrient broth (Himedia, Mumbai) and shaken

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for 24 hr at 28°C. Bacterial cells of 2 ml with 10^8 cfu ml^{-1} cells were inactivated by overnight soaking in 0.3% formalin (Song *et al*., 1992). Formalin killed bacterial cells were harvested by centrifugation at 11 000 x g for 25 min at 4°C, washed twice with PBS (pH 7.0). Different ratios (50, 100, 200 µl) of formalin killed bacteria without adjuvant were injected (Intraperitoneal) to three different size (5-10, 20-30, 35-50) of seabass and control sere as saline were maintained separately. Immunization was repeated 3 times at 2-week intervals. Two weeks after the last immunization, immunized seabass and control fishes were immersed in *V. anguillarum* suspension (1 x 10^5 cfu ml^{-1}) for 60 min. The infection mortality and survival (%) were calculated by Relative Percentage Survival index (RPS). RPS value is the survival rate of fishes after vaccination when compared with control fishes.

$$RPS = \frac{\% \text{ mortality rate in the vaccinated}}{\% \text{ mortality rate in the non vaccinated}} \times 100$$

Results and Discussion

In this present study, lethal dose LD_{50} for *V. anguillarum* was found to be 10^6 cells ml^{-1} after 24 hr of IP injection to seabass. The sign of infection was necrosis and haemorrhagic areas near the base of fins, exopthalmia and ulcers on the skin surface. Among those pathologies, Pasteurellosis and Vibriosis caused by bacterial pathogens *Photobacterium damselae* subsp. *piscicida* and *Vibrio anguillarum*, respectively, lead to the heaviest losses in aquaculture production of seabass (Afonso *et al*., 2005). Morbid fish after injection was sacrificed and fixed in preservative. Since teleost fish have no medullary cavity in their bones, the spleen and kidney serve as the primary haemopoietic organs (Agius and Roberts, 2003). As fish have no lymph nodes, the spleen alone plays an essential role in antigen trapping (Press, 1998). Melano-macrophages in the head-kidney, spleen and liver of sea bass and gilthead seabream have been investigated (Meseguer *et al.,* 1994). Therefore spleen plays a vital role in immune response in fishes and high production of melano-macrophages. Spleen was dissected and thin sectioned at 6 µm were stained by the standard hematoxylin and eosin. Thin section of control fish spleen showed that the intensified white pulp (WP) and red pulp (RP) areas (Fig. 1). *V. anguillarum* infected fish spleen has macrophages entrapping bacterial cells with high density (Fig. 2) which shown that the immune cells activated against the pathogens. However the lethal dose of bacterial cells overcomes the phagocytosis due to bacterial enzymes. Additionally, Noya *et al.* (1995) reported that experimentally infected gilthead sea bream *Sparus aurata* of different sizes with a highly virulent *P. piscicida* strain concluded that, in the largest fish, both macrophage and granulocytes may be involved in phagocytosis and killing of *P. piscicida*.

Since we used formalin killed vaccine through IP injection to different size range for sudden response. After two weeks of vaccination, immunized fishes of all size were immersed in seawater containing *V. anguillarum* suspension (1 x 10^5 cfu ml^{-1}) and unvaccinated fishes as control. During vaccination, a small rashes were found in injected area and automatically cured after two days except 5-10 g size fishes, it extend to third day. Gomes *et al*. (2006) reported that injection and immersion are the two major methods that have been developed for use at an industrial scale and in commercial production of salmonids and these methods have different advantages and disadvantages with respect to the level of protection, side effects, practicability and cost-efficiency. After two days, infections to unvaccinated were observed and the mortality rates were calculated as 100, 80 and 70% respectively to their sizes. Mortality due to typical histopathological lesions, such as necrosis and atrophy of hepatocytes, necrosis of sheathed arteries in the spleen and

Table - 1: Survival rate of vaccinated fishes and its Relative Percentage Survival (RPS)

S.No.	Size of fish (g)	Fish survival rate after 15 days		RPS (%)
		Control	Vaccinated	
1.	5-10	20/20	12/20	60
2.	20-30	4/20	16/20	75
3.	35-50	6/20	16/20	62.5

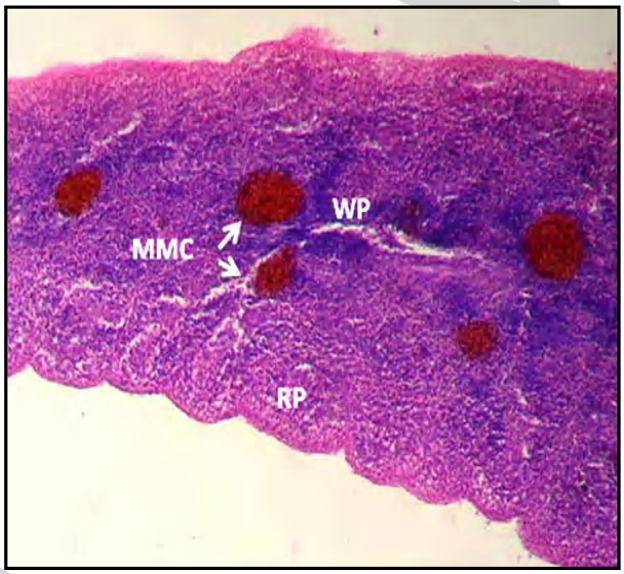

Fig. 1: Section of spleen of control Asian seabass (*Lates calcarifers*) showing white pulp (WP) and red pulp (RP) H&E X 400

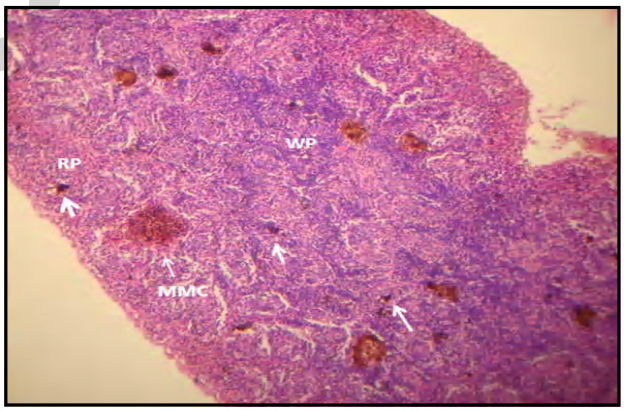

Fig. 2: Section of spleen of Asian seabass (*Lates calcarifers*) infected with *V. anguillarum* showing macrophages entrapping bacterial cells (Arrows), H&E X 600

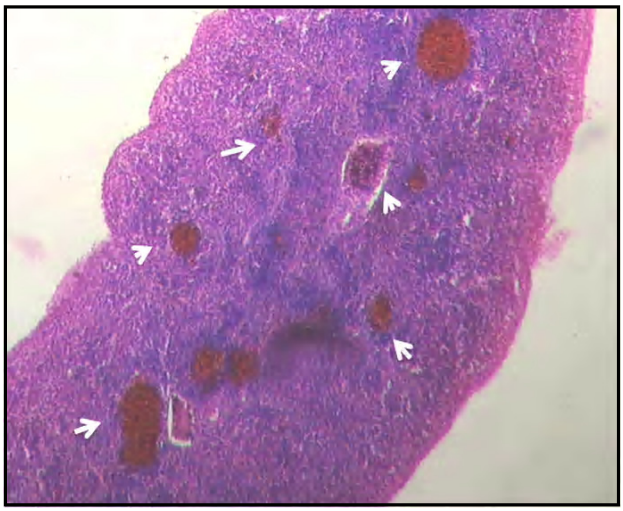

Fig. 3: Section of spleen of Asian seabass (*Lates calcarifers*) infected with *V. anguillarum* showing clusters of melanomacrophage centers (MMC) H&E X 600

necrosis of renal tubules and glomeruli in the kidney has occurred. Deivasigamani (2008a,b) stated that humoral and cell mediated immunity have the ability to endeavor immune response against sheep red blood corpuscles (SRBC) in cat fish and also the head kidney is the major antibody producing organ. However the RPS% for vaccinated fishes were 60, 75 and 62.5 respectively. This result shown that the vaccinations for 20-30 g fishes stay alive, when match up to the other sizes. Therefore IP injection for above 20 g fishes and immersion or dipping for below 10 g fishes is advisable to control *V. anguillarum* in fish ponds. Some other reports stated that to induce sufficient protective immune responses, antigens mixed up with feed and reach the lymphoid tissue of the hindgut without destruction during passage through the gastrointestinal tract (Rombout *et al.,* 1985; Davidson *et al.,* 1993; Jenkins *et al.,* 1994).

Kumar *et al.* (2007) reported that DNA vaccination with the major outer membrane protein (OMP encoding gene) from V. *anguillarum*, induces a significant humoral immune response and moderate protection against *V. anguillarum experimental* infection. However formalin killed bacteria vaccination could produce good survival rate and this procedures may useful to combat different bacterial pathogens.

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