VACCINE ANALYSIS
Chapter V – VACCINE ANALYSIS

5.1 Introduction

Vaccination of humans and other animals, including fish, is one of the major methods for preventing infectious disease (Potter and Baiuk, 2001). Immunisation primes the immune system of the host against pathogens encountered during infections (Thomson and Adams, 2004). Fish vaccination in the aquaculture industry has been considered to be very important in reducing economics losses caused by disease (Ellis et al., 1988; Rahman and Kawai, 2000 and Ebanks et al., 2004). Several kinds of vaccines have been investigated / developed against *A. hydrophila* including whole cell (WC), OMPs, ECPs, LPS and biofilms, although currently no commercial vaccine exists.

*A. hydrophila* is an important pathogen that has caused major loss in the aquaculture industry for decades (Shotts *et al*., 1972; Oliver *et al*., 1981 and Esteve *et al*., 1995). Many attempts have been made to develop an effective vaccine against *A. hydrophila* (Lamers *et al*., 1985; Baba *et al*., 1988; Leung *et al*., 1997 and Rahman and Kawai, 2000). The effect of number of inactivated WC vaccines has been reported. For example, An increase in serum antibody levels against *A. hydrophila* was showed in common carp immersed in a preparation of heat inactivated *A. hydrophila* (Lamers *et al*., 1985). Later, Kusuda *et al*., (1987) also found an increase in the concentration of total serum proteins, when common carp were immunised by injection, immersion and oral administration of killed *A. hydrophila* and these are shown to produce antibodies in their
serum, bile, skin and gutmucus, and skin and muscle extract. A polyvalent vaccine containing heat killed WC and formalin inactivated ECP of *A. hydrophila* has also been tested in two Indian major carp species (rohu and mrigal), but it failed to protect the fish against bacterial challenge (Chandran *et al.*, 2002). However these authors observed relatively high antibody titre in immunized fish suggesting that the low survival in the vaccinated group may be due to an impact of unknown stress on the fish in the pond, or maybe the antibodies induced by the vaccine were not protective.

Many factors must be considered for developing an effective vaccine. The vaccine produced should be protective and should not cause any adverse effect in the host. In addition, the vaccine should be cost effective for global use in aquaculture industry. World demand for high quality protein food has stimulated a rapid development of intensive fish culture techniques. Inherent intensive rearing of fish involves problems of high loaded densities, declining water quality, in adequate diets, handling and disease control. Man has only recently recognized the threat imposed by disease and its limitation on economics of the aquaculture industry (Roberts, 1978). When confronted with the disease problem, the producer has had essentially only two expensive and potentially devastating options at his disposal: a) antibiotic prophylaxis and treatment of fish (which could affect palatability of food and may result in the development of resistant bacteria); and b) destruction of all fish at the station followed by thorough hatchery disinfection and reintroduction of disease – free stock. Both the producer and the fish health specialist appreciate the presence of chemotherapeutants for controlling disease, the registered drugs and chemotherapeutants are scarce (Souter, 1983).
In recent years, a new technique for the prevention of fish disease is rapidly emerging as a result of research into the development of fish vaccine (Robertson, 2011). Fish immunology has a more recent history than human veterinary immunology but the techniques used are similar. However, methods of administering vaccines to fish differ and are dependent upon species, pathogen, temperature, and environment (Anderson, 1974). Immunity is an important physiological mechanism in animals for protection against infectious disease agents and the maintenance of internal homeostasis (Ingram and Alexander, 1980).

Prophylactic treatments and good management practices reduce the susceptibility to diseases. Although, antibodies can overcome bacterial diseases, consumer health and food safety issues prevent their use in aquaculture. Moreover, established viral diseases cannot be treated. Therefore, vaccination is the best alternative to combat bacterial and viral diseases. It has been a key tool in the success of the livestock, pig, poultry and salmon industries in the fight against infectious diseases. In salmon farming, the use of vaccines is now routine that all fish stocked in cages are vaccinated. However, in Asia, fish vaccination is still a new concept that is not very well known and understood by farmers and professionals working in the area. A vaccine can be either water or oil based. Typically, injection vaccines are oil based as the oil provides adjuvant qualities. This means that the oil increases the effectiveness of the vaccine as well as the duration of the desired protection.

The choice of vaccine depends on the particular case. It will depend on whether protection can be obtained, the duration of the protection possible versus the required
duration, the final cost of the vaccine in relation to the benefit to the farmer and the registration limitation imposed by authorities in the countries where the vaccine is marketed.

5.2. Materials and Methods

5.2.1. Immune response studies

5.2.1. a. Preparation of *A. hydrophila* culture for vaccine

A loop full of *A. hydrophila* culture was incubated overnight at 37°C. About 5 ml of culture obtained was inoculated in a 50 ml nutrient broth medium and incubated overnight at 37°C. The culture was then serially diluted and spread plated on nutrient agar medium and incubated overnight at 37°C for enumeration. The culture was simultaneously inactivated with heat treatment and incubated at 37°C for 48 hrs. About 1 ml of culture was then spread on nutrient agar plates and incubated overnight at 37°C to check for inactivation. The inactivated broth culture was then pelleted by centrifugation at 500 rpm for 10 min. The pellet was then suspended in saline and used as vaccine for immunization.

5.2.1. b. Preparation of vaccine adjuvant

Fifty ml of heat killed *A. hydrophila* vaccine was mixed with 50 ml of Montanide Adjuvant ISA 763 A VG as suggested by manufactures of adjuvants.

5.2.1. c. Vaccination

*Catla catla* fish fingerlings were dipped in to the adjuvanated vaccine for about 30 seconds.
5.2.1. d. Collection of blood from vaccinated fishes

Two fishes from each vaccinated tank and control tank were sacrificed on 3rd, 7th and 10th day respectively after vaccination.

5.2.1. e. Collection of serum from blood

Few microlitres of blood obtained from each fish were kept for blood smear and rest of the blood was subjected for serum collection. Blood was collected from fishes using sterile insulin syringe and introduced in to sterile eppendorf tube. A pinch of EDTA was added in to the eppendorf tube. Then the blood was centrifuged at 10,000 rpm for 10 minutes. The eppendorf tubes were incubated overnight in a slanting position at 4˚C. The supernatant was collected in a fresh eppendorf. The serum obtained was used to test the immune response.

5.2.1. f. Differential Leucocyte count

The blood cells were stained with Leishman’s strain to show the following appearance to differentiate the blood cells and also confirm the sample stained with Coomassive brilliant blue.

5.2.2. g. Polymorph nuclear Neutrophil

It shows a pinkish cytoplasm filled with nearly uniform, fine granules, which take a pink color. The nucleus is usually divided irregularly into five lobes which are commented by five lands. It is a round cell with a distinct nuclear membrane.
5.2.2. h. Eosinophil

It is distinguished by compact course granules with cosin, colour, circular shape, blobbed nucleus looks like spectacles.

5.2.2. i. Basophil

It contains prurplish granules which are usually intermediate in size between those of the proceeding tubes of cells and are less refractive then the eosinophil granules they tend to vary in size and depth of staining are often sq the nucleus stains more faintly and lobilation is often in distinct life 8-12 days.

5.2.2. j. Monocytes

It is larger than a large lymphocyte, the nucleus which appears like a kidney and twisted. The cytoplasm has a frostily appearance and has five granules size 18µ.

5.2.1. k. Agglutination Test

A clean grease free microscopic glass slide was taken. About 50 µl of serum collected from vaccinated fishes was smeared in the center of the glass slide. 50 µl of antigen and 50 µl of serum collected from vaccinated fishes were smeared in the centre of the glass slide. 50µl of serum alone was smeared on extreme right of glass slide. After incubating for 2 minutes, the slide was viewed under microscope using 40X magnification.

5.2.1. l. Latex Agglutination test

Ten µl of latex suspension was washed twice by adding 400µl of 0.05 M glycine saline buffer and centrifuged at 13,400rpm for 10 minutes. Pelleted latex was then
resuspended in 200µl of 0.05 M glycine saline buffer and 30µl of antigen solution was added to it. The suspension was mixed thoroughly for 30 minutes at room temperature. The latex was washed twice by adding 400µl of 0.05m glycine-saline buffer and centrifuge the latex at 13,400 rpm for 15 minutes. Then the latex was re suspended in 200µl of 0.05M glycine saline mix containing 0.1% of an irrelevant protein to block any remaining protein binding sites and stored at 4°C. 50 µl of antiserum was mixed with 50 µl of coated latex on glass slide. The slide was kept aside for 2 minutes and agglutination was read visually, illuminating the slide from the side against dark background.

5.2.2. Enzyme Linked Immunosorbant Assay

Hundred µl of antigen diluted with coating buffer was added to appropriate wells in the microtitre plate and incubated for a overnight at 4°C. The residual liquid was then tapped out. The wells were washed with wash buffer. 300 µl of blocking solution was added to each well and incubated for 20 minutes at room temperature. The wells and were emptied residual liquid was tapped out. The wells were then washed with wash buffer. Hundred µl of diluted serum was added to the first well and serially diluted in the subsequent wells. Wells were incubated at room temperature for one hour. After incubation, the residual liquid was washed out with wash buffer. 100µl of secondary enzyme-antibody conjugate was added to all wells and incubated for one hour at room temperature. Then the wells were washed with wash buffer. 100µl of substrate (tetra methyl benzene and H₂O₂) was added to each well and incubated for few minutes. After sufficient colour development 100µl of stop solution was added to each well. Finally
50 µl of sample from the well was subjected to absorbance determination at 490nm using a biophotometer (Eppendorf, Germany).

5.2.3. Experimental infection (Challenge study)

Three tanks containing 30 fishes in each were allowed to acclimatize well and was subjected to challenge study, the post vaccination. The fishes in two tanks were administered with vaccine with adjuvant, vaccine alone respectively. Fishes in the third tank were considered as control and were unvaccinated. All the fishes in three tanks were challenged with *A. hydrophila* (2X10⁹ CFU/ml).

5.3. Results and Discussion

The heat killed *A. hydrophila* vaccine and the adjuvant used for enhancing the immune response is shown in **Plate 5.1**. The *Catla catla* fishes were left free for over a period of two weeks in order to make the fishes to get adapted to the new environment. *Catla catla* fish fingerlings were dipped in to the vaccine for about 30 seconds as shown in **Plate 5.2**. Blood from the vaccinated fishes was collected from the gills of fishes using sterile insulin syringe.
Plate. 5.1. Preparation of vaccine substance using heat killed *A. hydrophila* and adjuvant

Plate. 5.2. Administration of vaccine substance to fish by Immersion method
The effects of heat killed antigen, vaccine, vaccine with adjuvant on the leucocyte count of fish *Catla catla* was studied and the results was shown in Fig. 5.1 to 5.6.

The differential counts of leucocytes such as lymphocytes, monocytes, eosinophils and basophils was determined and it was found to be higher in the fishes treated with the vaccine and adjuvant than the control and fishes treated with heat killed antigen and vaccine alone. The results also imply that the lymphocytes are the most dominant leucocyte type in the blood of *Catla catla*.

The neutrophils, eosinophils and basophils were the granulocytes present in the blood of *Catla catla*, the neutrophils were found to be reduced in the treated groups when compared with the control.

Microscopic observation of the diluted *A. hydrophila* isolate and the serum collected from the vaccinated fishes showed the clumps of antigen-antibody molecules confirming bacterial agglutination. Mixing of latex coated *A. hydrophila* with the serum collected from vaccinated fishes showed visual agglutination. The difference between plain latex suspension and agglutinated latex was clearly observed as shown in Plate 5.3. The blood collected from the vaccinated fishes stained with eosin and hemotoxylin is fish shown in Plate 5.4a and 5.4b. Increases in number of macrophages in vaccinated fish compared to the unvaccinated control were observed under oil immersion objective. The absorbance values obtained from biophotometer on analyzing the serum samples increased with the increase in the number of days after vaccination. The values recorded on 3rd, 7th and 10th day post vaccination is shown in Fig.5.7.
Fig. 5.1. Differential Leucocyte count in *C. catla* administered with heat killed antigen and control fish

![Graph showing differential leucocyte count over time.]

% of leucocytes

- Lymphocytes
- Monocytes

Heat killed antigen treated fishes

Fig. 5.2. Differential Leucocyte count in *C. catla* administered with heat killed antigen and control fish

![Graph showing differential leucocyte count over time.]

% of leucocytes

- Neutrophils
- Eosinophils
- Basophils

Heat killed antigen treated fishes
Fig. 5.3. Differential Leucocyte count in *C. catla* administered with vaccine with adjuvant and control fish

![Graph showing the percentage of Leucocytes over time for vaccinated and control fish.](image)

Fig. 5.4. Differential Leucocyte count in *C. catla* administered with vaccine and control fish

![Graph showing the percentage of Leucocytes over time for vaccinated and control fish.](image)
Fig. 5.5. Differential Leucocyte count in *C. catla* administered with vaccine and control fish

**% of leucocytes**

- **Lymphocytes**
- **Monocytes**

**Vaccine & adjuvant fishes**

Fig. 5.6. Differential Leucocyte count in *C. catla* administered with vaccine & adjuvant and control fish

**% of leucocytes**

- **Neutrophils**
- **Eosinophils**
- **Basophils**

**Vaccine & adjuvant treated fishes**
Plate 5.3. Latex agglutination test

Plate 5.4. a. Macrophages in vaccinated fish

Plate 5.4. b. Macrophages in control fish
Fig. 5.7. Antibody response in vaccinated fish determined by ELISA

Days Post Vaccination Vs. Absorbance Values

Absorbance Value

Days

Control Fish
Vaccinated Fish
Fishes in each category which were challenged with this pathogen, post vaccination, exhibited their own level of immune responses and the result was shown in Table 5.1.

**Table 5.1.** Experimental infection (Challenge study)

<table>
<thead>
<tr>
<th>S.no</th>
<th>Vaccine/Control</th>
<th>Total Fishes</th>
<th>Test Fishes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Alive</td>
</tr>
<tr>
<td>1</td>
<td>With Adjuvant</td>
<td>30</td>
<td>28</td>
</tr>
<tr>
<td>2</td>
<td>Without Adjuvant</td>
<td>30</td>
<td>18</td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
<td>30</td>
<td>3</td>
</tr>
</tbody>
</table>

*A. hydrophila* is an important bacterial pathogen as it infects both fish and humans (Janda and Abbott, 1996). *A. hydrophila* infection in fish culture results in decrease in production and economic losses. Use of antibiotics or chemicals to control *A. hydrophila* infections has an adverse effect on the environment and also the host developing resistant strains of bacteria (Shariff, 1998; Thayumanavan *et al.*, 2003).