Materials and Methods
4.0 MATERIALS AND METHODS

4.1 EXPERIMENTAL FISH

*Cirrhinus mrigala* fingerlings (Fig -1) of average weight 5.0±2.0 were purchased from Poondi Lake, Thiruvallur District, and Tamil Nadu. Fingerlings were brought to the laboratory with well aerated plastic bags (Fig -2). The fingerlings were maintained in 200 L Fibre reinforced plastic tanks. The water was replaced daily to maintain the quality of the water. Various environmental factors like temperature and oxygen were maintained properly throughout the experimental process and also the feed were given at proper intervals. Fingerlings were allowed to acclimatize for 15 days to meet the laboratory conditions and to assess their disease free health status. The experimental groups were subjected to a photo period of 12h light and 12h darkness. Fingerlings were fed with commercial pellet feeds (Taiyo, China) at a ratio of 10% of their body weight / day, during the experimental period.

4.2 BACTERIAL STRAIN

The virulent strain of *Edwardsiella tarda* (ATCC 15947) subculture obtained from Christian Medical College, Vellore, Tamil Nadu and *Pseudomonas fluorescens* (ATCC 13525) obtained from Government Medical College, Theni, Tamil Nadu were used in the present study.

4.2.1 BACTERIAL CULTURE OF EDWARDSIELLA TARDA

*Edwardsiella tarda* were cultured in Tryptic soy broth medium at 30°C for a period of 24 hours. In 24 hours of bacterial culture, the colonies were characterized and used for vaccine preparation (Fig -3 and 4).
4.2.2 BACTERIAL CULTURE OF PSEUDOMONAS FLUORESCENS

The Bacterial isolate (*Pseudomonas fluorescens* ATCC 13525) were inoculated on Nutrient broth medium at 28°C overnight and pH 7.5 was maintained for the growth. After 24 hours, Bacterial culture were characterized and used for vaccine preparation (Fig-5 and 6).

4.3 MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF *EDWARDSIELLA TARDA*

*Edwardsiella tarda* were confirmed by morphological and biochemical characterization tests according to Bergey *et al.*, 1984 and Holt *et al.*, 1997. The confirmation test of these bacteria was done with the help of selective media used for culturing that particular bacterium. Antimicrobial susceptibility test for *E. tarda* and *P. fluorescens* was done by Kirby-Bauer Method.

4.3.1 GROWTH ANALYSIS

4.3.1.1 TEMPERATURE TOLERANCE

The Tryptic soy broth medium was placed in to water baths of 25°C, 35°C and 40°C, 0.1 -1.0 ml of bacterial suspension was pipetted aseptically in to the test tubes and the liquid media was mixed with the suspension, incubated at the given temperature for 10 minutes then the inoculated medium poured in to sterile petri dishes. Then petri dishes were incubated at 28°C for one week. The bacterial growth observed and estimated the temperature tolerance of each strain by counting the colonies formed in the agar plate.
4.3.1.2 GROWTH ANALYSIS - pH TOLERANCE

The test tubes were labelled and 0.1-1.0 ml of the bacterial suspension was pipetted into Tryptic soy broth medium with pH 4, 6 and 10 under aseptic conditions and vortexed. The test tubes were incubated for 1 week and estimated the pH tolerance of each strain by examining the intensity of bacterial growth (observed the turbidity of each broth and compared with a control test tube that has not been inoculated with bacteria).

4.3.1.3 GROWTH ANALYSIS - NaCl TOLERANCE

Five ml volumes of Tryptic soy broth medium containing 1.5 and 4.0 (w/v) NaCl were inoculated and incubated for one week and observed the bacterial growth.

4.3.2 COLONY MORPHOLOGY

The loop of isolated bacterial culture was taken and inoculated to Tryptic soy agar medium and incubated at 30°C for 48 hours or until growth. Colony colour, size and texture were observed under oil immersion microscope 100 x magnifications.

4.3.3 GRAM STAINING

After the incubation period, a loop full of culture was taken from the Tryptic soy broth and it was smeared on the glass slide. Then they were dried in air and heat fixed for gram staining. Grams crystal violet was flooded over the smear for 1 minute and washed with water until it gets decolourised and washed with water. Finally safranin was added as a counter stain and washed with water. It was dried and observed under the oil immersion microscope (Magnification x 100).
4.3.4 MOTILITY TEST

The loop of isolated bacterial culture is taken and is inoculated to this Motility medium and incubated at 30°C for 48 hours or until growth was evident. Motility was determined at 48 hours liquid medium cultures and identifies the ability of bacteria to move was observed.

4.3.5 CATALASE TEST

This test is used to identify organisms that produce the enzyme, catalase. A loop full of culture was taken from the Tryptic soy broth and it was kept on the glass slide and a few drops of \( \text{H}_2\text{O}_2 \) were added into the culture and a change was observed.

4.3.6 HYDROGEN SULPHIDE TEST

This test was commonly performed to differentiate members of *Enterobacteriaceae* and it was used as confirmatory test of *E. tarda* bacterium. One slant was used as control and other TSI slant was used to inoculate with culture. Then, the Two TSI slants were incubated for 24 to 48 hours at 35 °C and colour change and gas production was observed.

4.3.7 CYTOCHROME OXIDASE TEST

This test is used to identify microorganisms that produce the enzyme cytochrome oxidase, capable of reducing oxygen. It is commonly used to distinguish between oxidase–ve *Enterobacteriaceae* and oxidase + *Pseudomonadaceae*. Filter paper was dipped in Kovacs oxidase reagent. A loop full of culture was taken from the slant and it was put on the filter paper and observed the colour change.
4.3.8 CARBOHYDRATE FERMENTATION TEST

Kligers iron agar is a differential medium and commonly used to separate glucose fermenting members and lactose non fermenting members of the family Enterobacteriaceae. It tests for an organism’s ability to ferment the glucose and lactose to acid (pyruvic acid) in to gaseous by products. The isolated bacterial colony was inoculated onto agar medium and incubated for 18 hours at 35°C until the bacterial growth was evident.

4.3.9 SIMMONS CITRATE TEST

Simmons Citrate agar was used to test the ability of the bacteria that uses citrate as a source for energy utilisation. Then the isolated bacterial culture was streaked in to the Simmon’s Citrate agar slant and it was incubated at 37°C for 24 hrs. After incubation period, the colour change was observed.

4.3.10 NITRATE TEST

This test was important in the identification of Gram-negative species. Nitrate broth was used to determine if an organism was capable of reducing nitrate (No$_3$) to nitrite (No$_2$) or other nitrogenous compounds via the action of nitrogenase enzyme. For this test nitrate broth is required and after the incubation of bacteria, nitrate reagents A (sulfanilic acid) and B (naphthylamine) wooden sticks for zinc powder were used. The isolated bacterial colony was inoculated to the nitrate broth and incubated then 6-8 drops of nitrite reagent A was added followed by nitrite reagent B and the reaction was observed. Then small amount of zinc powder was added at the end of a wooden stick and then the reaction was observed.
4.3.11 **VOGES PROSKAUER TEST**

The isolated culture was inoculated into 5ml of MR/VP medium and it was incubated for 24h at 35°C. After incubation, 3ml of $\alpha$-naphthal solution and 1ml of potassium hydroxide solution were added. Finally, the color change was observed.

4.3.12 **INDOLE PRODUCTION TEST**

The indole test was performed on well isolated colonies and a loop full of suspected culture was taken from the slant and was inoculated into an indole broth. Then the indole broth containing the isolated culture was further incubated for 35-37°C for 24 to 48 hours. After incubation, 10-12 drops of Kovac’s reagent was added to the test broth and shaken gently. The color development was observed within 3-5 minutes.

4.3.13 **METHYL RED TEST (MR)**

For this test MR/VP broth (pH 6.9) and Methyl red solution, 0.02% was prepared. A loop full of selected culture was inoculated into the MR/VP broth and incubated at 37°C for 24hrs. After 24 hours of incubation, 6 drops of methyl red indicator added to broth culture and the color change was observed.

4.3.14 **ANTIMICROBIAL SUSCEPTIBILITY TEST FOR *E. TARDA* (KIRBY-BAUER METHOD)**

The bacteria were tested for antimicrobials susceptibility test by using Disc-Diffusion technique. The antibiotic namely, erythromycin, novobiocin, ampicilin, erythromycin, and tetracycline were tested in vitro against *E. tarda*. In
this method, a culture is spread on an appropriate medium. A pre-determined concentration of an antimicrobial are placed onto the infected agar plate (Petri dish). During incubation, the agent diffuses from the disc, creating a concentration gradient that decreases with the distance from the discs. After 24 hours, incubation at 25°C sensitivity was observed on the basis of the size of the inhibition zone (with no microbial growth) around each disc. Following the procedure of Toranzo et al., (1985) a strain was considered resistant to a compound when no inhibition was produced, which indicates whether the microorganism was resistant or sensitive to the antibiotic.

4.4 MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION PROCEDURE OF PSEUDOMONAS FLUORESCENS

*Pseudomonas fluorescens* was confirmed by morphological and biochemical characterization tests according to Bergey et al., 1984 and Holt et al., 1997. Antimicrobial susceptibility test for *P. fluorescens* was done by Kirby-Bauer Method.

4.4.1 GROWTH ANALYSIS

4.4.1.1 TEMPERATURE TOLERANCE

The nutrient broth medium was placed in to water baths of 25 °c, 30 °c and 40 °c, 0.1 -1.0 ml bacterial suspension was pipetted aseptically in to the test tubes and the liquid media was mixed with the suspension, incubated at the given temperature for 10 minutes then inoculated medium poured in to sterile petri dishes. Then petri dishes were incubated at 28 °c for one week. The bacterial growth was observed and estimated the tempearture tolerance of each strain by counting the colonies formed in the agar plate.
4.4.1.2 **GROWTH ANALYSIS -pH TOLERANCE**

The test tubes were labelled and Pipetted 0.1-1.0 ml of the bacterial suspension into nutrient broths with pH 6 and 7.5 under aseptic conditions and vortexed them. The test tubes were incubated for 1 week and estimated the pH tolerance of each strain by examining the intensity of bacterial growth (observe the turbidity of each broth and compare it with a control test tube that has not been inoculated with bacteria).

4.4.1.3 **GROWTH ANALYSIS -NaCl TOLERANCE**

Five ml volumes of nutrient broth containing 0%, 5% and 7% (%w/v) Nacl were inoculated with bacterial strain and incubated for one week and observed the growth.

4.4.2 **COLONY MORPHOLOGY**

The loop of isolated bacterial culture is taken and inoculated to nutrient agar medium and incubated at 28°C for 48 hours or until growth. Colony colour, size and texture were observed under oil immersion microscope (100x magnifications).

4.4.3 **GRAM STAINING**

After the incubation period, a loop full of culture was taken from the nutrient broth medium and it was smeared on the glass slide. Then they were dried in air and heat fixed for gram staining. Grams crystal violet was flooded over the smear for 1 minute and washed with water until it gets decolourised and washed with water. Finally safranin was added as a counter stain and washed with water. It was dried and observed under the oil immersion microscope.
4.4.4 MOTILITY TEST

The loop of isolated bacterial culture is taken and is inoculated to this Motility medium and incubated at 28°C for 48 hours or until growth was evident. Motility was determined at 48 hours liquid medium cultures and identifies the ability of bacteria to move was observed (ie, flagellated cells).

4.4.5 CATALASE TEST

This test is used to identify the organisms that produce the enzyme, catalase. A loop full of culture was taken from the nutrient broth medium and it was kept on the glass slide and a few drops of H₂O₂ were added into the culture and a change was observed.

4.4.6 HYDROGEN SULPHIDE TEST

This test was commonly performed to differentiate members of Enterobacteriaceae and it was used as confirmatory test to determine the presence of specific bacterium. One slant was used as control and other Triple Sugar Iron agar slant was used to inoculate with culture. Then, the TSI slants were incubated for 24 to 48 hours at 35 °C and colour change and gas production was observed.

4.4.7 CYTOCHROME OXIDASE TEST

This test is used to identify microorganisms that produce the enzyme cytochrome oxidase, capable of reducing oxygen. This test will distinguish aerobic vs. anaerobic metabolism. Filter paper was dipped in Kovacs oxidase reagent. A loop full of culture was taken from the slant and it was put on the filter paper and observed the colour change.
4.4.8 SIMMONS CITRATE TEST

Simmons Citrate agar was used to test the ability of the bacteria that uses citrate as a source for energy utilisation. Then the isolated bacterial culture was streaked into to the Simmon’s Citrate agar slant and it was incubated at 37°C for 24 hrs. After incubation period, the colour change or until the bacterial growth was observed.

4.4.9 NITRATE TEST

This test was important in the identification of Gram-negative species. Nitrate broth was used to determine if an organism was capable of reducing nitrate (No$_3$) to nitrite (No$_2$) or other nitrogenous compounds via the action of nitrogenase enzyme. For this test nitrate broth is required and after the incubation of bacteria, nitrate reagents A (sulfanilic acid) and B (naphthylamine) wooden sticks for zinc powder was used. The isolated bacterial colony was inoculated to the nitrate broth and incubated then 6-8 drops of nitrite reagent A was added followed by nitrite reagent B and the reaction was observed. Then small amount of zinc powder was added at the end of a wooden stick and then the reaction was observed.

4.4.10 VOGES PROSKAUER TEST

The isolated culture was inoculated into 5ml of MR/VP medium and it was incubated for 24h at 35°C. After incubation, 3ml of α-naphthal solution and 1ml of potassium hydroxide solution were added. Finally, the colour change was observed.
4.4.11 ANTIMICROBIAL SUSCEPTIBILITY TEST FOR

PSEUDOMONAS FLUORESCENS (KIRBY-BAUER METHOD)

The bacteria were tested for antimicrobials susceptibility test by using Disc-Diffusion technique. The antibiotic namely, penicillin, chloramphenicol, erythromycin, Kanamycin and neomycin were tested invitro against *P. fluorescens*. In this method, a culture is spread on an appropriate medium. A pre-determined concentration of an antimicrobial are placed onto the infected agar plate (Petri dish). During incubation, the agent diffuses from the disc, creating a concentration gradient that decreases with the distance from the discs. After 24 hrs incubation at 25°C sensitivity was observed on the basis of the size of the inhibition zone (with no microbial growth) around each disc. Following the procedure of Toranzo *et al.*, (1985) a strain was considered resistant to a compound when no inhibition was produced, which indicates whether the microorganism is resistant or sensitive to the antibiotic.

5.0 VACCINE PREPARATION

The whole cell vaccine and outer membrane protein vaccine were prepared by the method of Thangaviji *et al.*, 2013 with minor modifications. Four types of monovalent and bivalent vaccines were prepared from the WC and OMP vaccines.

5.1 TYPES OF VACCINE (*Edwardsiella tarda, Pseudomonas fluorescens*)

1. Monovalent WC Vaccine (Formalin inactivated).
2. Monovalent OMP Vaccine.
3. Bivalent WC Vaccine (Formalin inactivated).
4. Bivalent OMP Vaccine.
5.1.1 PREPARATION OF MONOVALENT WHOLE CELL VACCINE (FORMALIN INACTIVATED)

The Whole cell vaccine is prepared from the harvested bacterial cultures in Tryptic soy broth (E. tarda) and Nutrient broth medium (P. fluorescens) at 28°C to 30°C for 24 hours followed by inactivation of using 0.6 % formalin. The formalin was removed by spinning the culture at 1500 x g (20 min) at room temperature and washed with 0.85% saline and finally re suspended in saline to a concentration of 1x10^8 cells/ml. This is collected and stored at 20°C until it is used for the experimental work (Fig -7 & 8).

5.1.2 BIVALENT WC VACCINE (FORMALIN INACTIVATED) / MIXED WC VACCINE (E.TARDA + P.FLUORESCENS)

The two monovalent formalin inactivated vaccines (E. tarda and P. fluorescens) were mixed with equal volume to form the final volume (1:1=1x10^8 cells/ml) of Bivalent WC vaccine.

5.1.3 MONOVALENT OUTER MEMBRANE PROTEIN (OMP) VACCINE

The 24 hours Bacterial culture were harvested by centrifugation from Tryptic soy broth (E. tarda) and Nutrient broth medium (P. fluorescences) at 3000 x g [20min at 25°C]. The cell pellets were washed twice in phosphate buffer saline (PBS) and once in 10 mM Tris-hydrochloride [pH7.5]. The cells were re-suspended in Tris-HCl and sonicated at 50w for 30s (4 times on ice). After sonication, the suspension was mixed with sarkosyl for solubilization of the outer membrane protein and incubated at 25°C for 30mins. After incubation, the suspension was centrifuged at 4000 x g (20 min) and the supernatant were collected and again
centrifuged at 45,000 x g (45 min) for collection of the pellets and finally the pellets were dissolved in 100 µl of distilled water. This OMP pellets was stored at 20°C until it is used for the experimental work (Fig -7 & 8).

5.1.4 BIVALENT OMP VACCINE / MIXED OMP VACCINE (E.TARDA + P.FLUORESCENS)

The two Monovalent OMP vaccines (E. tarda and P. fluorescence) were mixed with equal volume to form the final volume (1:1= 33ug/ ml) of Bivalent OMP vaccine.

5.2 STERILITY TEST OF THE VACCINES

This test was done as described by Aly, (1981) by cultivation of the prepared bacterins or vaccines on Tryptic soy broth to ensure that there is no growth of E. tarda or other pathogens. This method was repeated for WC and OMP vaccines of P. fluorescens.

5.3 PREPARATION OF IMMUNO ADJUVANT

In most of the vaccines, the adjuvant is a crucial ingredient for efficacy. Recently, the herbal Immuno adjuvant (A. racemosus) has been shown to improve the vaccine delivery against aquatic pathogens. A. racemosus tuber powder was obtained from Aringhar Anna siddha college, Arumbakkam (Fig -9). The herbal immuno adjuvant prepared by following the method of Gautam et al., 2004. The A. racemosus tuber powders were extracted with hot water at 100 °C for 2 hours. The extracts are filtered (Fig -10) and supernatants were condensed, (by using a rotary evaporator at 55 °C) lyophilized and stored at 4 °C (Fig -11). The extracts contained steroidal saponins possessing immune adjuvant properties.
5.4 QUALITATIVE PROTEIN ANALYSIS OF PREPARED VACCINES – SDS-PAGE METHOD

The prepared WC and OMP proteins were resolved by 10% SDS-PAGE (Laemmli, 1970) to generate protein profiles. It offers a rapid and accurate way to determine the protein molecular weights. A 50 µl of each proteins sample (WC and OMP of *E. tarda*) were analysed by SDS-PAGE with the discontinuous buffer system according to the method of Laemmli (1970). The microfuge tubes were labelled as culture, whole cell, outer membrane protein and marker respectively and added 50 µl of sample solubilizing buffer to each tube and heated at 100°C for 3 to 5 minutes. A 50 µl of each sample was loaded in to a 5% stacking gel (10% SDS - 60 µL, 10% APS -60 µL, TEMED - 6 µL) and 10% separating gel (10% SDS - 30 µL, 10% APS -30 µl, TEMED - 3 µL) with a thickness of 1.5 mm. Electrophoresis was carried out with a Mid-range molecular weight marker for 1 hour at 100 volts. After electrophoresis, proteins in the gel were stained with the coomassie stain (30 minutes). After 30 minutes destainer was used. Then removed destaining solution by rinsing the gels twice with distilled water and the protein bands were visualized. Same method was repeated to estimate the molecular weight in the sample of WC and OMP proteins of *Pseudomonas fluorescens*.

5.4.1 BAND ANALYSIS OF SDS-PAGE OF *EDWARDSIELLA TARDA* AND *PSEUDOMONAS FLUORESCENS*

SDS-PAGE result was analysed by G image software analyser. Protein bands of *E. tarda* and *P. fluorescens* was analysed to determine the molecular weights of WC and OMP Proteins of both bacterial pathogens.
5.5 QUANTITATIVE PROTEIN ANALYSIS OF PREPARED VACCINE – LOWRY'S METHOD

The prepared WC and OMP vaccines were re-suspended in PBS and the protein concentration was estimated by using Lowry's method. This method is commonly used to determine the total protein concentration of the sample. Protein was estimated by the method of Lowry et al., (1951) using Bovine Serum Albumin (BSA) as the standard. The test tube with 1ml distilled water was served as blank. A volume of 0.2 ml to 0.6 ml of BSA as working standard in 5 test tubes and made up to 1ml using distilled water. A volume of 0.2 ml of the test sample (WC and OMP) was made up to 1.0 ml with distilled water and 4.5 ml of reagent I (48 ml of 2% Na₂CO₃ in 0.1 N NaOH, 1 ml of 1% Na K Tartrate and 1ml of 0.5 % CuSO₄ 5 H₂O in H₂O) was added to all the tubes and incubated for 10 minutes. After 10 minutes 0.5 ml of reagent II (1Part Folin – Phenol (2 N): 1 part water) was added and incubated for 30 minutes. The end product of this reaction has a blue colour. The amount of proteins present in the sample was estimated at 660 nm in a UV-visible spectrophotometer and the standard graph was prepared. The amount of protein present in the given sample (WC protein and OMP of Edwardsiella tarda) was calculated from the standard graph and same method was repeated to calculate the proteins present in the sample of WC protein and OMP of P. fluorescens.

6.0 METHOD OF VACCINATION

6.1 IMMERSION METHOD (BATH VACCINATION)

In bath vaccination, fingerlings were immersed for 30 mins in diluted vaccine in separate vaccine tanks in the concentration of 1 part of vaccine in 10 volume of tank water (1:10) = 10⁸ cells / mL according to Mcintosh and Austin, 1993.
6.2 EXPERIMENTAL DESIGN

Fingerlings are divided into ten groups and each tank contains 50 fishes. One Blank group, one control group and eight experimental groups for immersion method (bath immunization). Feeding was stopped 24 hours prior to experimentation. Faster fingerlings suffer less handling stress and respond better. The primer dose was given on day 1 and booster dose was given on day 30 of the experimental period. The blank control groups have unvaccinated fingerlings without bacterial challenge. The control groups comprises of unvaccinated fingerlings and with bacterial challenge. The 8 experimental groups (monovalent WC, monovalent WC+A, monovalent OMP, monovalent OMP+A, bivalent WC, bivalent WC +A, bivalent OMP and bivalent OMP +A) of both pathogens received both vaccine delivery and bacterial challenge (Fig-12 and Table-1). The monovalent vaccination process was also done in monovalent *Edwardsiella tarda* and monovalent *Pseudomonas fluorescence* experimental groups.

6.3 VACCINE DELIVERY AND IMMUNIZATION

Fingerlings were vaccinated twice by using immersion method during the Experimental period. Prior to immersion in the diluted vaccine, the fingerlings were immersed in a hyper osmotic solution of NaCl (2% w/v) for 5 min (Fig -13&14). The primer dose is delivered on day 1, and the booster dose is delivered on day 30 of the Experimental period. The dosage of outer membrane protein vaccine was 33ug/ml and dosage of whole cell vaccine was at a concentration of 1 x 10^8 cells/ml. The immuno adjuvant concentration was 500 ug (Thangaviji *et al.*, 2012) added to the antigenic proteins. The experimental groups also treated by immuno adjuvant with antigens to form a volume of 1:1. Vaccination process done in separate vaccine tank (Fig -15) and after the vaccination, the fish were washed and returned to their original tank. The process of vaccination was repeated for all groups (Monovalent and Bivalent vaccines with or without adjuvant).
6.4 BACTERIAL CHALLENGE STUDY

After 30 and 60 days of post vaccination (dpv), 25 fingerlings from each group were challenged or experimentally infected with virulent *E. tarda*, *P. fluorescence* and mixed pathogens separately (Table-2) by bath challenge method (1:10 lethal concentration of $10^8$ cells/ml) outlined by Lillihaug, 1989. The Monovalent and Bivalent vaccine with or without adjuvanated fingerlings were observed for Relative percent survival, mortality rate, and other pathological signs for 10 days.

6.4.1 CHALLENGE WITH VIRULENT *EDWARDSIELLA TARDA*

After 30 and 60 days of post vaccination (dpv), 25 fingerlings from Monovalent *Edwardsiella tarda* WC and OMP vaccine with or without adjuvanated groups and unvaccinated control group were challenged or experimentally infected with virulent *Edwardsiella tarda* strain.

6.4.2 CHALLENGE WITH VIRULENT *PSEUDOMONAS FLUORESCENS*

After 30 and 60 days of post vaccination (dpv), 25 fingerlings from Monovalent *Pseudomonas fluorescens* WC and OMP vaccine with or without adjuvanated groups and unvaccinated control group were challenged or experimentally infected with virulent *Pseudomonas fluorescens* strain.

6.4.3 CHALLENGE WITH BIVALENT/MIXED VIRULENT *EDWARDSIELLA TARDA* + *PSEUDOMONAS FLUORESCENS*

After 30 and 60 days of post vaccination (dpv), 25 fingerlings from Bivalent *Edwardsiella tarda* and *Pseudomonas fluorescens* WC and OMP vaccine with or without adjuvanated groups and unvaccinated control group were challenged
or experimentally infected with virulent strains of *Edwardsiella tarda* and *Pseudomonas fluorescens* (mixed pathogens).

7.0 **PATHOLOGICAL SYMPTOMS**

The behavioural and external pathological symptoms of *Edwardsiellosis* and Pseudomonas septicemia were observed after the experimental infections of virulent *E. tarda*, *P. fluorescens* and mixed pathogens separately for 10 days. The varying degree of susceptibility was interpreted as due to the strain virulence of the bacterial pathogens tested and susceptibility of the fish species.

8.0 **RELATIVE PERCENT SURVIVAL**

The survival and mortality rate was recorded up to 10 days post challenge and the relative percent survival in each group is calculated by the following formulae (Amend, 1981).

\[
RPS = 1 - \frac{\% \text{ of mortality in vaccinated group}}{\% \text{ of mortality in unvaccinated group}} \times 100
\]

9.0 **MORTALITY RATE**

After the bacterial challenge mortality rate was recorded at every 24 hours interval for 10 days.

10.0 **IMMUNOLOGICAL STUDIES**

10.1 **COLLECTION OF BLOOD SAMPLES**

After 30 and 60 days post vaccination, fingerlings were randomly selected from the control and experimental groups for blood collection. Fingerlings were anaesthetized with clove oil (Sipra *et al.*, 2014) at 50 µl/L of water before collecting
blood from fish. Whole blood was drawn from cardiac puncture (Swain et al., 2007) by using 1ml Insulin syringe rinsed with 2.7% EDTA solution (Fig. -16). The collected blood was immediately transferred to tube coated with thin layer of EDTA (as anticoagulant) and shaked well in order to prevent haemolysis and clotting of blood (Fig. -17, 19 and 21). This blood sample was used for Leucocyte count. For separation of serum, the blood samples were transferred to sterile eppendorf tubes (Fig -18, 20 and 22) without anticoagulant and allowed to clot for 2 hours at room temperature in a slanting position. After 2 hours, blood samples were centrifuged at 3000 x g for 15 min and stored at -20 °C until used for antibody titer or agglutination test. Same method was used to collect the blood samples from all the experimental groups. The test samples and controls were analysed in triplicates.

10.1.1 BLOOD LEUCOCYTE COUNT

A sample of 1 µl whole blood is mixed with 20 µl of a weak acid (2 % Glacial acetic acid) solution in a clean test tube and was shaken well and kept for two minutes to suspend the cells uniformly in a solution and a drop of leishmans stain was added. The 2 % Glacial acetic acid lyses mature red blood cells, which hemolyzes mature erythrocytes and facilitates leukocyte counting. The standard dilution for leukocyte counts is 1:20. This dilution is prepared using the leukocyte Unopette system. The dilution is mixed well and incubated to permit lysis of the erythrocytes. Following the incubation period, the dilution is mounted on a hemacytometer. The cells are allowed to settle and then are counted in specific areas (4 large Squares) of the hemacytometer chamber under the microscope. For this technique following regaents and equipment were used as follows: Two leukocyte Unopette reservoirs; each containing diluent, Glacial acetic acid, two Unopette capillary pipettes, Hemacytometer (Neubauers counting chamber) with cover glass
and microscope. The total Leucocyte count was calculated by following formulae (Rusia and Sood, 1992).

\[
\text{Number of cells (Cu mm}^{-1}) = \frac{\text{Number of cells counted x dilution}}{\text{Area counted x depth of fluid}}
\]

Average of cells = the average of the total number of cells counted in the four large squares on both sides of the hemacytometer.

Dilution =20
Area counted = 4
Depth of fluid=0.1mm

10.1.2 SERUM ANTIBODY TITRE (AGGLUTINATION TEST)

Serum antibody titer was measured using the agglutination protocol described by Swain et al., 2007. The agglutination test was conducted in ‘U’ shaped microtitre plates. Bacterial strains were grown in respective medium for 24 h at 28°C, harvested by centrifugation at 2000 x g for 15 min, and washed with PBS. Bacteria were washed in phosphate buffered saline (PBS) twice more before adjusting the bacterial suspension concentration to each separated bacteria to 1.0 × 10^9 CFU of bacterial cell/ml. The mrigal sera (15 µl) raised against different bacterial antigen (Monovalent and Bivalent) were serially two-fold diluted in PBS (pH 7.2) and then equal amount of bacterial suspension were added separately to each well. The plates were kept overnight at room temperature (28°C) and agglutination titer was calculated. Plates were incubated at room temperature overnight prior to microscopic examination (X40) for agglutination (Nayak et al., 2004). The titer was calculated as \( \log_2 \) of the reciprocal of the highest dilution of serum showing complete visible agglutination of the bacteria. Similarly, the
agglutination titer of sera raised against mixed bacterial antigens to mixed bacteria was also calculated. Positive (vaccinated serum) and negative (normal serum) were included in each plate as controls.

11.0 STATISTICAL ANALYSIS

The Leucocytes and antibody titer values were subjected to a one-way analysis of variance (ANOVA) and the significance of the differences between means was tested using Duncans multiple range test (DMRT). All the Statistical analysis was carried out by using SPSS 16 software.