Material and Methods
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3.1. Experimental animals

Juveniles of *F. indicus* (7.23 ± 2.14 cm) were collected from a cooperative farm in Vypeen Island and brought to Central Marine Fisheries Research Institute. The animals were stocked in one ton fibre glass tanks at the rate of 30 numbers per tank. The tanks were provided with continuous aeration. Water salinity was maintained at 25 ppt. The animals were acclimatized to laboratory conditions for a minimum period of one week. Shrimp were fed with commercial pellet feed at the rate of 3% body weight, daily. Feacal matter and uneaten feed were siphoned out, and 75% of the water was replaced daily. Injured and weak shrimp were discarded.

3.2. Haematological studies of *F. indicus*

3.2.1. Cellular factors

3.2.1.1. Characterization of haemocytes

3.2.1.1.1. Light microscopy

The method followed was of Mix and Sparks (1980) with modifications. The animals were blotted dry of water. From each shrimp, 100 μl of haemolymph was withdrawn from the heart. A 2 ml syringe containing freshly prepared, cold, 10% seawater-formalin (1.4 ml), attached to 26 guage needle was used. The syringe was shaken and rotated gently and the contents transferred to an eppendorf tube. The sample was kept at 4°C for 1-3 hours for proper fixation. The haemolymph was centrifuged at 8000 rpm for 5 minutes at 4°C. The supernatant was decanted. The cell pellet was mixed with two drops of supernatant and thick smears were prepared on clean, grease-free glass slides. They were air dried, post-fixed in absolute methanol for 5 minutes, rinsed in distilled water and stained with dilute Wright’s stain of pH 7.2 for 5-10 minutes. The scum was washed off with distilled water and the slides were dried. The smears were viewed under a light microscope. The cells were classified on the basis of presence or absence of granules and their staining reactions.
3.2.1.1.1. Cell dimensions

Measurements were made in Wright's stained smears. Triplicates of 200 stained cells from three slides were measured using a calibrated ocular micrometer. The cell size was determined by measuring the largest and shortest axis excluding pseudopodia. Nuclear size was estimated by measuring the largest and shortest nuclear diameter. Measurements were taken for all the cells.

3.2.1.1.2. Transmission electron microscopy

The method of Martin and Graves (1985) was modified for ultrastructural studies of *F. indicus* haemocytes. A 26-gauge needle attached to a 1 ml syringe was used to withdraw 0.1 ml haemolymph from the heart. The syringe contained 0.4 ml chilled fixative (3% glutaraldehyde in 0.1M sodium cacodylate buffer containing 12% glucose, pH 7.8). After thorough mixing, the sample was transferred to an eppendorf vial and immediately kept at 4°C for 2 hours. The fixed sample was centrifuged at 8000 rpm for 5 minutes at 4°C. The pellet was given three washes with 0.1 M sodium cacodylate buffer (pH- 7.8, containing 24% sucrose). It was post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 2 hours at 4°C. The sample was centrifuged at 8000 rpm for 5 minutes and the pellet was washed three times in 0.1 M sodium cacodylate buffer (pH 7.8 containing 24% sucrose). Two percent agarose (HiMedia Ltd) gel was prepared in cacodylate buffer (pH- 7.8) containing 24% glucose. The molten gel was cooled to 60°C and added to the eppendorf vial in a water bath at 60°C. The sample was mixed thoroughly and allowed to solidify. The solidified gel was cut into tiny pieces of 1 mm thickness and dehydrated in an ascending series of acetone. The sample was further processed for electron microscopy. It was infiltrated and embedded in Spurr's (1969) low viscosity resin. Thin sections of 60-90 nm were cut on an LKB-Ultramicrotome, stained with uranyl acetate and lead citrate and viewed in a Hitachi-H600 Transmission electron microscope.
3.2.1.3. Cytochemical analysis

Various cytochemical studies were conducted in the haemocytes of *F. indicus*. Cytochemical staining methods were employed to demonstrate carbohydrates, lipids and the enzymes prophenoloxidase, acid phosphatase and myeloperoxidase in haemocytes.

3.2.1.3.1. Periodic Acid-Schiff's (PAS) reaction

The presence of carbohydrates in the haemocytes of *F. indicus* was studied using the PAS method of Sanders (1974). Haemolymph was drawn into a 2 ml syringe containing citrate/EDTA buffer, pH 7.3. The cells were concentrated by centrifugation at 8000 rpm at 4°C for 15 minutes. Haemocyte smears were made on clean grease-free glass slides. These were fixed with methyl alcohol and incubated in 0.5% periodic acid for ten minutes. The smears were rinsed with distilled water and treated with Schiff's reagent for 15 minutes. After this, they were placed in 3 changes of sulphurous acid rinse (10 ml of 1 N HCl in 210 ml of 0.05% Na HCO₃) for 3 minutes each. The smears were left in running water for five minutes and rinsed in distilled water. They were stained with Harris haematoxylin for 30 to 45 seconds, after which washes were given in running water till the smears turned blue.

3.2.1.3.2. Demonstration of lipids

The method of Sanders (1974) was used to test the presence of lipids in *F. indicus* haemocytes. Air-dried smears were placed in formalin vapours for ten minutes. The fixed smears were rinsed in running water, after which they were layered with Working Sudan Black B solution for 1 hour. The smears were washed in running water for 2 to 3 minutes. After blotting dry, they were counterstained with Maygumwald-Giemsa stain and viewed under a microscope.

3.2.1.3.3. Prophenoloxidase activity

Haemocyte smears were fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH- 7.4) for 1 minute at 4°C and washed three times (for 15
minutes each) with 0.1 M sodium phosphate buffer (pH 7.4). The slides were then incubated in 0.1% L-dopa (dihydroxyphenyl alanine) prepared in 0.1 M phosphate buffer with 2% NaCl for 90 minutes at 30°C (Smith and Soderhall, 1983) and counterstained with dilute Giemsa.

3.2.1.1.3.4. Acid phosphatase activity

As per the method described by Sanders (1974), air-dried smears were fixed in formalin-acetone (20% formalin in 50% acetone) for 1 minute at 0°C and rinsed in running water. The fixed smears were incubated at 37°C for 1 hour in a freshly prepared and filtered acid phosphatase substrate solution containing Fast Blue BBN. The slides were then rinsed with distilled water, air dried and counterstained with neutral red solution for 3 minutes. Control slides were also prepared by incubation with stock solution without Fast Blue BBN.

3.2.1.1.3.5. Myleoperoxidase activity

Air dried smears were fixed in 10% alcoholic formalin for 60 seconds, rinsed in distilled water for 15-20 seconds. Slides were then incubated in myleoperoxidase incubation mixture (100 ml of 30% ethyl alcohol, 0.3 g of benzidine dihydrochloride, 1 ml of 0.132 M zinc sulphate, 0.7 ml of 3% hydrogen peroxide, 1.5 ml of 1N sodium hydroxide and 0.2 g of Safranin O) according to the method of Sanders (1974) for 30 seconds, washed briefly in running tap water, dried and counterstained with Giemsa.

3.2.1.2. Total haemocyte count (THC)

Twenty micro litre of haemolymph was drawn from the heart of the shrimp with a 26 gauge needle into a 2 ml syringe containing 1 ml cold 10% seawater formalin (Mix and Sparks, 1980). After thorough mixing it was transferred to an eppendorf vial and kept at 4°C for 1 hour. The cell suspension was loaded into a haemocytometer and the total haemocyte count was determined per millilitre of haemolymph (Perazzolo and Barracco, 1997).
3.2.1.3. Differential Haemocyte Count (DHC)

From each animal, 20 μl haemlymph was drawn into a syringe with 1 ml fixative (Mix and Sparks, 1980) and the contents stored for 1 hour at 4°C. This was centrifuged at 8000 rpm for 5 minutes. From the concentrate, thick smears were prepared on glass slides and stained with dilute Wright's stain of pH 7.2. From each slide, 200 cells were observed under oil emulsion objective (1000X) and the percentage of each cell calculated.

3.2.1.4. Phagocytic activity of haemocytes

Fifty micro litre of haemolymph was drawn into 1 ml of anticoagulant (cold, freshly prepared citrate/EDTA buffer, pH-7.3 (Le Moulac et al., 1997) and K-199 medium, pH-7.3 (Itami et al., 1994) taken in the ratio 1: 1. The cells were separated from the haemolymph by centrifugation at 8000 rpm at 4°C for 10 minutes and washed three times with the medium. The cells were supplemented with fresh K-199 medium, mixed gently and layered on clean glass cover slips. These were incubated at 25°C for 30 minutes and the cells were allowed to adhere to the coverslips. The non-adhered cells were washed off gently with K-199 medium. Then the cover slips were layered with killed yeast cells (1×10^8 cells/ml) suspended in K-199 medium. The layered cover slips were incubated at 25°C for 90 minutes, washed gently with medium and fixed with absolute methanol for 5 minutes. They were washed with medium and stained with Wright’s: Giemsa (1:1) stain, pH-6.8. The stain was washed off with distilled water, dried and mounted on glass slides. Two hundred cells were counted from each slide and the percentage phagocytosis calculated as follows,

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\text{Percentage phagocytosis} = \left( \frac{\text{number of haemocytes ingesting yeast cells}}{\text{total number of haemocytes observed}} \right) \times 100.
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3.2.1.4.1. Nitro Blue Tetrazolium reduction assay

The production of O_2^- during oxygen burst was detected using Nitro Blue Tetrazolium reduction assay. The method followed was of Anderson et al. (1991) with modifications. Phagocytosis was performed according to the above
procedure. The excess yeast on the cover slips was washed off with PBS (pH-7.4) and the cover slip air-dried. The cover slip was covered with filtered NBT solution (0.2% in pH-7.4 PBS) for 30 minutes and kept in a humid chamber. The excess stain was decanted off, washed, air dried and fixed in methanol. It was mounted in DPX on a clean glass slide and observed under the microscope.

3.2.2. Humoral factors of the haemolymph

The parameters studied were the serum protein profile of serum i.e., total serum protein concentration, the serum polypeptide profile, haemagglutinating activity and activity of different enzymes viz. phenoloxidase, acid phosphatase and alkaline phosphatase.

3.2.2.1. Preparation of serum

Haemolymph drawn from the heart of shrimp without anticoagulant was quickly transferred to an eppendorf vial and allowed to clot at 4°C for 4 hours. The serum was separated from the clot by centrifuging at 8000 rpm at 4°C for 10 minutes and used for studying various humoral factors.

3.2.2.2. Protein profile of serum

3.2.2.2.1. Total serum protein concentration

The total protein concentration was determined using the method of Lowry et al. (1951). Bovine serum albumin was used as the standard.

3.2.2.2.2. SDS-PAGE of serum

The serum proteins were separated by SDS-PAGE by the method described by Laemmli (1970). Electrophoresis was carried out on 10% separating gel. One microlitre of serum sample was diluted 100 times with double distilled water. The samples were mixed with equal volumes of sample buffer, boiled for 5 minutes and loaded in the wells along with standard protein marker (Genei, India). Electrophoresis was carried out at 140v for 5-6 hours and the gel was stained with Coommasie brilliant blue.
3.2.2.3. Haemagglutination

Haemagglutination was performed in 96 well 'v' bottom micro titer plates following the method of Imai et al. (1994). Serial double dilutions of the serum (25 μl) were made using 0.85% Na Cl containing 5 mM CaCl as diluent. Haemagglutinating activity was carried out using 2% (v/v) chicken RBC suspension (25 μl) in phosphate-buffered saline. The plate was allowed to stand for 1 h at room temperature and for 24 h at 4°C. The agglutination titer was recorded as the reciprocal of the maximum dilution giving positive agglutination.

3.2.2.4. Enzyme profile of haemolymph

3.2.2.4.1. Phenoloxidase activity

The method of Preston and Taylor (1970) with modifications was followed. To 2 ml of 0.01 M dihydroxyphenyl alanine (DOPA) in Tris-HCl buffer (0.05M, pH 7.5), 0.2 ml of enzyme source was added. Two hundred micro litre of sodium dodicyl sulphate in 0.05M Tris buffer (mg/ml) was added as activator. The increase in O.D. of the sample was recorded up to 3 minutes for every 30 seconds interval at 420 nm. The results were expressed as enzyme units (0.001 change in O.D./minute/mg of protein). The total protein was estimated using the method of Lowry et al. (1951).

3.2.2.4.2. Acid phosphatase

The method followed was of Varley (1980). Ten microlitre of serum was diluted 10 times with phosphate buffered saline (pH 7.3). It was allowed to react with 0.01 M disodium phenyl phosphate and citrate buffer in 1:1 proportion at a pH of 4.9. The phenol released was allowed to react with 0.6% of amino-antipyrene in 2.4% Ferricyanide solution in 0.5N sodium hydroxide and 0.5N of sodium bicarbonate. The O.D was recorded at 510 nm. The amount of phenol released per 100 ml serum was determined from a standard curve using known amount of phenol. The results were expressed in KA units (mg phenol released/100 ml serum/ h).
3.2.2.4.3. Alkaline phosphatase

Twenty five microlitre of serum was diluted to 50 μl with phosphate buffered saline (pH 7.3). The enzyme activity was estimated using a commercially available diagnostic kit (Sigma Diagnostics, India Ltd, Product No. 72011, according to King and King (1954). The O.D. was measured at 510 nm and enzyme activity was expressed in KA units.

3.3. Immunomodulation in *F. indicus*

3.3.1. Exposure to *V. parahaemolyticus*

3.3.1.1. Estimation of sub-lethal dose

A pathogenic strain of *V. parahaemolyticus* species isolated from diseased shrimp was used for experimental infection. Dilutions in phosphate buffered saline with a cell count of $10^7$ cells/ml, $10^6$ cells/ml, $10^5$ cells/ml, and $10^4$ ml was chosen to determine the 24 hour lethal concentration. For each concentration 30 shrimps were maintained in triplicates. The animals were injected intramuscularly with the respective dosages on the fifth abdominal segment. A bacterial count of $10^5$ ml was determined as the lethal dose.

3.3.1.2. Experimental infection

Seventy animals were maintained in 1 ton tanks, each for control and test groups. A bacterial cell density of $10^6$ cells/ml was used as the sub-lethal dose (1/10th of the lethal dose). Each shrimp was inoculated with 0.1 ml of the bacterial suspension in PBS on the fifth abdominal segment. The control group was injected with 0.1 ml of PBS per animal. 10 shrimps, each from test and control groups were sampled after 30 minutes, 3 hours, 1 day, 2 days, 3 days, 1 week and 2 weeks. Heamolymph collected from the heart was pooled. Shrimp samples were also fixed in Davidson’s fixative for histological studies.
3.3.2. Exposure of *F. indicus* to organophosphate pesticide, nuvan

The organophosphate pesticide used for the study was dichlorvos, known by the trade name nuvan. Initial experiments were conducted to determine the LC$_{50}$ of the compound.

3.3.2.1. Estimation of LC$_{50}$ of Nuvan for *F. indicus*

The commercial preparation of dichlorvos, nuvan, marketed by Syngenta India Ltd., was used. The concentration of active ingredient was 1000 mg/ ml. The 96 h LC$_{50}$ of nuvan for the experiment was determined as per the method of Reish and Oshida (1987). One millilitre of nuvan was added to 100ml of distilled water, so that 1 ml of the solution contained 10 mg of nuvan. The concentrations selected for calculating 96 h-LC$_{50}$ were 0.2, 0.1 and 0.05 ppm of nuvan. Ten animals were kept in 50 liters of 25 ppt seawater for each test concentration. They were maintained in triplicates for a week. A control was also maintained in triplicates. Complete water exchange was made everyday of the experiment and the chemical concentration maintained. The moralities were plotted on a logarithmic graph and 50% of mortality concentration was identified from the graph. Thus the 96-hour LC$_{50}$ was found to be 0.1 ppm.

3.3.2.2. Experimental exposure

The animals were given 1 week of acclimatization. The animals were exposed to nuvan at 0.02 ppm, 0.05 ppm and 0.08 ppm concentration, respectively. Each dose was tested in triplicates reared as groups of 10 animals. The control group was also maintained. Twenty-five percentage and 75 % water exchange was made on alternate days, during which the concentration of the chemical in the water was maintained. The animals were given pelleted feed at 3% body weight, twice a day. The experiment period lasted 2 weeks. Samples were taken after two weeks. Pooled heamolymph was used for analysis. For histological studies, samples were fixed in Davidson’s fixative.
3.3.3. Exposure of *F. indicus* to heavy metal, zinc

To study the effect of exposure to the heavy metal zinc in shrimps, animals were exposed to sub-lethal concentrations of zinc. As per the report of Viswanathan and Manisseri (1995) the 96 h LC$_{50}$ of zinc for *F. indicus* juveniles is 1668.1 ppb, i.e; 1.6681 ppm. Hence a sub-lethal concentration of 0.1 ppm was selected as the test concentration.

3.3.3.1. Preparation of heavy metal salt solution

The chemical used for the experiment was zinc sulphate (ZnSO$_4$. 7H$_2$O, M.W. - 287). A stock solution of 3 ppm zinc was prepared by dissolving 6.63 g of zinc sulphate in 500 ml of distilled water. A calculated amount was added to the experimental tubs, containing known volume of seawater, to get the desired concentration of 0.1 ppm zinc in the medium.

3.3.3.2. Experimental design

*F. indicus* juveniles were stocked at a rate of 6 animals per 30 l of 25 ppt diluted seawater. Triplicates were maintained. The tubs were provided with ample aeration. A control group maintained in triplicates was also kept. The animals were fed on commercial pellet feed *ad libitum*. Eighty percent of the rearing medium was renewed every day and the required amount of heavy metal maintained. The experimental period was for 2 weeks. At the end of two weeks, the pooled haemolymph from the animals of each replicate was analyzed. On termination of the experiment shrimp samples were fixed in Davidson’s fixative for histology studies.

3.3.4. Exposure of *F. indicus* to salinity variation

Juveniles of *F. indicus*, after an acclimation period of 5 days, were transferred to experimental tanks (100 l) with continuous aeration. Salinity levels of 3 ppt, 10 ppt and 25 ppt were chosen for the experiment. Ten animals per tank were maintained in triplicates in diluted seawaters of respective salinities. The animals were slowly acclimatized to the respective salinity. All the groups were
fed with a commercial pellet feed at a rate of 3% average body weight. Fecal matter and food waste were siphoned out every day. A water exchange of 50%, and complete water exchange on alternate days was made during the entire experimental period of two weeks. The haematological parameters were analyzed from the pooled haemolymph.

3.3.5. Effect of immunostimulant on the defense factors of *F. indicus*

The animals (6.5±2.1 g) were stocked at a density of 45 animals in 1 ton capacity tanks after an acclimation period of 1 week. The salinity of the tank water was maintained at 25 ppt and continuous aeration was provided. Control groups were maintained under the same conditions. Shrimp were fed with commercial pellet feed. The feed for the test group was incorporated with 'Allways', a commercial immunostimulant (Matrix BioScience India Ltd) containing β-1,3glucan, at a rate of 0.002g/1000g feeds. The test animals were fed at a rate of 2% of the body weight per day. The control animals received the same feeding regime, but without the immunostimulant. The tanks were cleaned of uneaten feed and faecal matter, and the water exchanged, every day. The experiment was carried out for a period of one month. Shrimp samples were collected weekly intervals. The pooled haemolymph was used for analysis. Samples were fixed in Davidson's fixative for histological analysis.

3.4. Statistical Analysis

The soft wares, Windows SPSS.10. and Systat 7.0.1 were used for statistical analyses. The results were analyzed using Analysis of Variance technique at 5% level of significance. The means were compared using Duncans multiple range test. Two sample t-test was used to test the significance for zinc exposed animals as also between the treatment means in bacterial and immunostimulant treatments.
3.5. Histology

The cephalothorax of *F. indicus* was dissected, fixed and processed according to the procedure of Bell and Lightner (1988). Specimens were fixed in Davidson's fixative and transferred to 50% ethyl alcohol after 24 hrs. The tissues were decalcified with 10% EDTA, dehydrated in ascending series of alcohol, cleared in xylene and embedded in paraffin wax (HiMedia,ltd). Trimmed blocks were cut into thin sections of 5-6 microns and mounted on clean, grease-free glass slides and mounted in DPX. The sections were stained using Haematoxylin and Eosin and examined under Leica (DMLS 60) triocular photomicroscope and photomicrographs were taken. Gills, heart, hepatopancreas, haematopoietic tissue, and antennal gland of the samples were studied.