Materials & Methods

3.1. Selection of Ponds

Three ponds which have a previous record of outbreak of EUS in different areas of Darjeeling and Jalpaiguri district, West-Bengal, India were selected for collection of EUS affected fishes. One pond is situated at by the side of North Bengal University campus, Darjeeling district. Two ponds are situated at Sahudanghi and Lataguri of Jalpaiguri district.

The measurement of the ponds at Sahudanghi (Fig.1), Lataguri (Fig.2) and by the side of the North Bengal university campus are 80`×90`, 40`×30` and 60`×40` respectively.

3.2. Collection and maintenances of healthy fish

Healthy *Cirrhinus mrigala* were collected from ponds of Darjeeling and Jalpaiguri districts having no past record of EUS outbreak. The health conditions of the fishes were given the utmost priority while selecting the fishes.

All the fishes were brought to laboratory in such a way so that the fishes were exposed to least possible stress. The healthy fishes were kept in a glass aquarium measuring 90×35×35 cm in which depth of water was 20-25 cm with constant blowing of air through water by air pump in laboratory. The fish were fed regularly with Tokyu fish food. All the fishes were acclimatized under laboratory conditions for at least 15 days before using them for experimental works.

3.3. Collection of diseased fishes

A number of fish species, e.g. *Cirrhinus mrigala, Catla catla, Labeo rohita, Puntius sp.* and *Mystus sp.* (Figs. 3, 4 and 5) were commonly affected in the ponds. Diseased fishes were collected from the infected ponds as well as from local markets.

Fig.1. Picture of the ponds of Sahudangi of district of Jalpaiguri.

Fig.2. Picture of the pond of Lataguri of district of Jalpaiguri.


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Fig. 3: Picture of a EUS affected *Catla catla* where the tail region has been totally lost.

Fig. 4: Picture of a EUS affected *Cirrhinus mrigala* where the ulcer has been so deep that the peritoneal cavity has been exposed.

Fig. 5: Picture of a EUS affected *Labeo rohita* showing ulcer on its body.

Fig. 6: Picture of intra peritoneal injection of a *Cirrhinus mrigala.*


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3.4. Materials for histopathological techniques

The internal organs such as thymus, head kidney and spleen of healthy and diseased fish, *Cirrhinus mrigala* were taken out from the fishes using sterilized dissecting instruments. In case of diseased fish ulcer tissue was also taken out using sterilized dissecting instruments. The collected samples were fixed in Bouin's fixative and preserved in cedar wood oil if necessary.

Bouin's fixative

a) Saturated aqueous picric acid solution – 75 mL

b) Formalin – 25 mL

c) Glaceial acetic acid – 5 mL

3.5. Materials for Tissue-Imprinting technique

Materials for Graham-Knoll Benzidine counterstained with Giemsa-

1. Giemsa solution

   a) Giemsa Powder -1 gm

   b) Glycerine – 66 mL

   c) Methanol – 66 mL

2. Formal Alcohol

   a) 40% Formalin – 5 mL

   b) Rectified spirit – 45 mL

3. Benzidine solution

   a) 90% alcohol – 30 mL

   b) Benzidine powder-1 pinch

   c) H_2O_2 - 7drops

   d) Distilled water – 20 mL

3.6. Materials for Periodic acid Schiff (PAS) staining (de Tomasi, 1936)

1. Solution A

   a) 10% Periodic acid

2. Solution B or Schiff's reagent

   a) Basic Fuchsin -1 gm

   b) Distilled water – 200 mL

   c) Hydrochloride acid (N) – 20 mL

   d) Sodium Metabisulphite -1 gm

   e) Activated charcoal – 2 gms

3.7. Materials for Haematological studies

1. Hayem's Solution

   a) Sodium chloride (NaCl) – 1%

   b) Sodium thiosulphate (NaS_2O_4) – 2.5%

c) Mercuric chloride ($Hgcl_2$) – 0.25%

2. N/10 Hydrochloric acid or HCl

Dissolve 10ml concentrated HCl with 1000ml distilled water.

3.8. Materials for Immunological studies

1. Alsever's Solution

a) Dextrose - 20gm

b) Sodium citrate - 8gm

c) Sodium chloride – 4.1gm

d) Double distilled water – 1000mL

2. Tris – NH_4Cl (0.84%, P_H 7.4)

a) Tris base – 206mg

b) Ammonium chloride (NH_4Cl) – 770mg

c) Distilled water – 100ml

3.9. Materials used for preparation of tissue samples for Transmission Electron Microscopy (TEM)

1. 0.1M Phosphate Buffer Solution (pH 7.2)

a) 0.1M disodium hydrogen phosphate – 72 mL

b) 0.1M sodium dihydrogen phosphate - 28 mL

2. 2% Para formaldehyde solution.

3. 2.5% Gluteraldehyde solution (EM grade).

4. 1% osmiumtetraoxide.

5. Acetone.

6. Toluene.

7. Araldite (CY 212)

8. Dodecenyl succinic anhydride (DDSA).

9. 2, 4, 6 tridimethyl amino methyl phenol (DMP-30).

10. Dibutylopthalate.

11. Uranyl acetate.

12. Lead citrate.

3.10. Histopathological techniques

3.10.1. Sampling and fixation

Live fishes were quickly put into a container of benzocaine solution (25mg/litre) for two minutes. Then the fish was taken out of the solution and pinned on a dissection tray. The external lesions were first excised from the fish body with sterilized scissors and forceps and put in Bouin's fixative. The internal organs were carefully dissected out, cut into small pieces of 3mm to 5mm and placed in fixative as early as possible. The volume of fixative was always at least 20 times of the volumes of the tissues. The tissues were kept in fixative for overnight.

3.10.2. Processing

The fixative was first washed out of the samples by 70% alcohol. Then routine procedure was followed by passing through a graded series of alcohol solutions (70%, 90% and 100%) for dehydration of the samples. (Schäperclaus, 1986)

After dehydration the tissues were then placed in xylene and infiltrated with molten paraffin (melting point 58° -60°C) and allowed to cool and harden. Some times tissues were preserved in cedar wood oil following fixation and dehydration.

3.10.3. Sectioning

The hardened paraffin containing the tissues were trimmed into rectangular blocks and mounted on the microtome and sections of 6μ thickness were cut. The sections were placed on grease free slides with albumin serving as an adhesive. The sections were properly stretched on warm distilled water and the slides were dried by keeping them overnight at room temperature.

3.10.4. Staining

Before staining the stretched and dried sections were completely dewaxed by keeping them in xylene for 5 to 10 minutes.

3.10.5. Procedures for Haematoxylin-Eosin stain

After complete dewaxing the slides with stretched sections were kept in absolute alcohol for 5 min. and passed through descending grades of alcohol e.g. 90%, 70%, 50% and 30% to water. The sections were then stained with haematoxylin and washed thoroughly with water to remove the excess stain. Next the sections were examined under microscope to check the differentiations level. If the differentiation was upto the mark, the sections were then passed through ascending grades of alcohol and stained with alcoholic eosin. The excess eosin was washed off by 90% alcohol and again examined under the microscope to check the differentiation level. If the counter staining was satisfactory the tissues were further dehydrated in absolute alcohol, cleared in xylene and mounted in DPX mountant.

3.11. Haematological techniques

3.11.1. Smear preparation and staining of blood

At first the slides on which blood smears were to be prepared, were made grease free. To make the slides grease free, the slides were first kept in chromic acid for 48 hours after which the slides were thoroughly washed with tap water and dried and then placed in alcohol and benzene (1:1) solution. Then the slides were wiped with a piece of clean cotton cloth and drawn through flame (Schäperclaus, 1986). Now a fresh drop of blood obtained by cutting the caudal region of a fish was placed on a grease free slide. A thin film of the blood was drawn with the help of another slide held at 45°C. The smears were then air dried at room temperature. The smears were then stained with Leishman stain, PAS, Graham-Knoll-benzidine counter stained with Giemsa and Sudan Black-B so that blood cell morphology can be characterized with sheer perfection.

3.11.2. Blood Sampling

Fishes were picked up from aquariums and put in benzocaine solution of 25mg/L for 2 minutes for proper anesthetization. The caudal region of the fish was cut off and the blood was collected in a tube rinsed with EDTA solution (1mg/mL). Leucocyte and erythrocyte counting and measurement of haemoglobin content of blood were done immediately after collection of blood.

3.11.3. Blood studies

Leucocyte count

The clean and dry W.B.C pipette was first rinsed with 1% sodium citrate so that blood clotting can be avoided. Blood was then sucked upto the 0·5 mark and immediately blood was diluted with Hayem's solution (NaCl- 1%; NaS_2O_4- 2·5%; $HgCl_2$- 0·25%) 20 times sucking upto 11 mark. Then the pipette was rotated slowly so that blood could be mixed properly with diluting solution. After discarding the clear fluid in the capillary part, a drop of mixture was added to the edge of a cover slip which was already placed on the platform of the haemocytometer (Fein-optik, Blakenburg, Germany). After three

minutes count was made under a microscope. The leucocytes were recognized by their refractile appearance.

Calculation

$$\text{Number of leucocytes per cubic mm} = \frac{\text{Number of cells counted} \times \text{Dilution} \times 10}{\text{Number of 1 sq.mm counted}}$$

Erythrocyte count

The clean and dry erythrocyte pipette was rinsed with 1% sodium citrate to avoid clotting of blood. Blood was then sucked to the 0·5 mark. The excess blood sticking to the tip of pipette was wiped off. Then the diluting solution was sucked upto 101 marks. The rubber tube was discounted and the either end of the pipette was gripped between forefinger and thumb and was shaken thoroughly for a minute. The rubber tube was reattached to the pipette and unused diluting fluid present in the stem of the pipette was rejected. Now an optimum drop was placed between the crevice of cover slip and the slide of counting chamber. Counting was then done in cells in 16 squares in five different parts of the slide.

Calculation

R.B.C of 80 small squares is multiplied by 10,000.

Determination of Haemoglobin content of blood by Sahli's method

Five drops of N/10 HCl were poured into an absolutely clean Sahli's tube so that the surface of acid reached the 10 mark in the tube. Fish blood was drawn into Sahli's pipette from cut off arterial caudalis upto the 20 mark. After wiping out the blood from the tip of pipette with the help of sterilized cotton, the tip of the pipette was dipped into the Sahli's tube below the surface of the HCl and the blood was carefully expelled. Drop by drop distilled water was added into the tube and was constantly matched with the colour of mixture with the colour of adjacent tubes on either side of the instrument. This was continued until the colour of the mixture exactly matched with the standard colour. Now the end point was recorded.

3.11.4. Quantifying erythropoietic efficiency of haemopoietic organs

Erythropoietic efficiency of haemopoietic organs was studied following the method of Homechowdhury and Jha (2001). The whole head kidney and the thymus were first dissected out from the live fish and excess peripheral blood was removed by using Whatman filter paper. Now both the organs were weighed and transferred to two separate watch glasses containing 1ml Hayemen's solution and were cut into several pieces. Cells were then dispersed by gently holding pieces of tissue with fine forceps in diluting fluid for exactly six minutes. Different cells along with blast cells were freed from the tissue and precipitated in the watch glass. After proper mixing of the cells in diluting fluid, 100mL of mixture was micropipetted into a dry and clean centrifuge tube and centrifuged for 1 minute at 1000 r.p.m. As the blast cells remained in the supernatant and other debris were pelleted. Ten microlitres of supernatant was carefully transferred to a Neubaeurs's improved double haemocytometer for total counting of different blast cells. At this stage special care was taken to avoid the mixing the debris to the testing sample. The temperature during tissue handling process was strictly maintained at 18°c. Presence of different blast cells as for example small lymphoid haemoblasts, basophilic erythroblasts, polychromatophillic erythroblasts, acidophilic erythroblasts and other lineage cells such as young reticulocytes, mature reticulocytes and erythrocytes in the haemocytometer were confirmed by comparing their morphometry with that of the various cell types identified from simultaneous tissue imprints of head kidney and thymus prepared according to Ashley and Smith (1963).

Calculations

The number of blast cells present in four corners of haemocytometer with an area of 1mm and a depth of 1/10 mm. So the volume would be $1/10mm^3$. Summation volume of four corners would be $= 4\times 1/10mm^3$

$$= 0\cdot 4\ mm^3$$

Now the number of blast cells/mm^3 was multiplied by dilution factor 2·5.

3.11.5. Preparation of tissue-imprints of head kidney, thymus and spleen following Ashley and Smith (1963)

The head kidney, thymus and spleen were dissected out. Excess blood associated with the organs was removed by blotting with Whatman filter paper. Next one end of the tissue was held by fine forcep, and with the help of a fine scissor the tissue was cut longitudinally into two parts and the cut was made at right angle to the tissue. Now the surface which was opened due to longitudinal cutting was put on the grease free slide, pressed gently and dragged for a distance resulting a thin film of tissue layered on the slide. The slides were now kept at room temperature for overnight. Then the slides were stained with appropriate staining method.

3.11.6. Staining procedures adopted for blood smears

1) Leishman's stain

1. The air-dried blood slide was kept in a modified petridish meant for staining of slides.

2. The slide was flooded with Leishman's stain, kept at 4°c.

3. The slide was kept for 5 minutes in covered condition.

4. After 5 minutes the same amount of distilled water was added to the slide.

5. The slide was then kept in covered condition for another 10 minutes.

6. The slide was washed with distilled water and air-dried.

7. Then the slide was mounted with D.P.X mountant.

2) Graham's Knoll- benzidine counterstained with Giemsa

1. Slides were first immersed in a cuplinger full of Formal alcohol for only 30 seconds.

2. The slides were kept in running water for 5 minutes.

3. The slides were air-dried.

4. The slides were then kept in Benzidine solution for 10 minutes

5. Then the slides were kept in running water for 10 minutes.

6. The slides were again air-dried.

7. The slides were kept in Giemsa (1mL Giemsa + 49mL distilled water) solution for at least 48 hours at 4°c.

8. Finally the slides were washed with distilled water and dried and mounted with D.P.X mountant.

3) Periodic Acid Schiff stain (De Tomasi, 1936)

1. The blood slides were slowly brought to water following down grades of alcohol (100%, 90%, 70%, and 50%).

2. Then the slides were kept in 10% periodic acid for 5-8 minutes.

3. The slides were washed with running water for 3 minutes.

4. The slides were washed with distilled water for 1 minute.

5. The slides were then kept in Schiff's reagent for 15 minutes in dark.

6. The slides were washed with tap water for 10 minutes and then dried and mounted with Euparol mountant.

4) Sudan Black – B

1. Slides were fixed in formalin vapour for 2-5 minutes.

2. Then slides were immersed in 25% acetic acid for 2 minutes.

3. Slides were washed thoroughly in tap water, then in distilled water.

4. The slides were then dried.

5. The slides were then stained in saturated Sudan Black B in 70% alcohol for 2 hours.

6. Excess stain was removed by washing in 70% alcohol.

7. Slides were blotted to dry and mounted in Glycerin jelly.

3.12. Immunological studies

Healthy *C. mrigala* (60-80gm) were carefully dissected for the location of their lymphoid organs and were placed in a petridish containing phosphate buffer saline (PBS – pH 7·2).

3.12.1. Antigen

Sheep blood was drawn from Juglar vein of sheep with the help of sterilized syringe in Alsever's solution and was washed twice with PBS. The pellet was diluted with PBS to make 25% solution of sheep red blood corpuscles or SRBC. This 25% solution of sheep red blood corpuscles or SRBC was used for immunization for all experiments.

3.12.2. Immunization

Different dosage of 25% SRBC and different routes of administration of SRBC were tried for experiments in search of the optimum dose and most effective route of administration out of three different doses (such as 0.1mL; 0.2mL and 0.4mL) 0·2ml was found to be the optimum and out of three routes of administration (such as intramuscular (i.m), intravenous (i. v) and intraperitoneal (i.p) injection was found most effective. (Fig. 6)

3.12.3. Cell Suspension

Healthy *C. mrigala* (60-80gm) was anesthesized by keeping in benzocaine solution. Lymphoid organs (head kidney, spleen and thymus) were dissected out separately and the

cells were dissociated by pressing the organs against a 200 gauge stainless steel wiremesh as per standard methodology (Chaudhuri, 1983). Before pressing the lymphoid organ against the wiremesh a small amount of PBS was injected into the lymphoid organ. At the time of rubbing, PBS was added drop by drop and the whole solution was collected in a sterilized Petri dish. The cell suspension was transferred to a centrifuge tube with the help of a Pasteur pipette. The centrifuge tube was spinned at 1000 r.p.m for 10 minutes in a centrifuge machine. The supernatant was discarded. Then 5ml chilled Tris-NH_4Cl (0.84%, pH 7.4) was added to the tube and kept for 5 minutes to lyse the red blood cells completely. The cell suspension was washed with PBS twice. The cell suspension was layered over Ficoll Hypaque solution and was spinned at 3000 r.p.m for 5-8 minutes. Lymphocytes were collected from interface and were washed twice with PBS. Finally cell suspension was adjusted to a required concentration with the help of Trypan blue exclusion test.

3.12.4. Haemogglutination Titre Assay

Healthy *C. mrigala* (60-80gm) was immunized by injecting 0.2mL of 25% SRBC intraperitoneally. After a certain day the caudal region of the immunized fish was cut and blood was collected in a small test tube. The blood was allowed to clot. After proper clotting it was centrifuged and the serum was collected. The serum was then heat inactivated for 30 minutes at 56°c to inactivate the complement. Round bottom 96 well microtitre plate (Tarson) was taken and 360µl of PBS was poured in well no.1, and 180µl of PBS was poured of the wells. 20µl heat inactivated serum was added in well no.1 and was mixed thoroughly. Then 200µl of mixed solution from well no.1 was taken and mixed with well no.2 and from well no.2 again 200µl of mixed solution was taken and was mixed with well no.3. This same method was followed upto well no.12 and from 12^{th} well the 200µl was taken and thrown out. Finally, 20µl of 1% SRBC was added to each well. The micro titre plate was kept at 37°C in a moist sterilized condition for overnight.

3.12.5. Plaque Forming Cell (PFC) Assay (Cunningham & Szenberg, 1968)

For PFC assay Cunningham and Szenberg (1968) technique with minor modifications was followed. Lymphocyte cell suspensions from head kidney and thymus on 5th day after immunization with an intraperitoneal injection with 0.2ml of 25% SRBC were prepared The concentration of cell in cell suspension was adjusted to 10^6 cells /mL. Prior to the preparation of cell suspension Plaque Forming Cell Assay (PFC) slides were prepared.

Preparation of PFC assay slides

1. At first the microscopic glass slides were cleaned and made grease free.

2. Clean and dry grease free slides were kept either on a smooth surfaced hard board or on a thick paper.

3. Three double strips of double-sided tape (TOV tape) cut at 7mm width were placed at the two ends and on the middle of the slides.

4. Then the cover of the upper surface of upper layer of the double sided tape was stripped off.

5. Two slides were placed on top of the exposed gum of the tape, matching each slide face to face.

6. Then the slides were pressed with the rubber stopper of a bottle along the taped line to get a set of two slides fixed face to face.

Charging of the PFC assay slide

First 0.1mL of the diluted cell suspension, 0.05mL of rabbit complement and 0·05ml of 10% SRBC were mixed in a small tube with a microtip pipette. Then the mixed solution was introduced into the microchambers of PFC assay slide. The longer edges of the paired slide were dipped into a molten 1:1 paraffin and Vaseline mixture to

seal the edges. The slides were then incubated at 37°C for 3-4 hours in a humidified atmosphere. After 3-4 hours of incubation in a humidified atmosphere haemolytic plaques were appeared as clear circular spaces on the background of intact red blood corpuscles. The plaques were counted under a low power microscope.

3.12.6. Transmission Electron Microscopy (TEM)

Head kidney, spleen and thymus were dissected out from healthy and EUS affected *Cirrhinus mrigala* (150gm) and cut into pieces of 2×1mm size. The pieces were washed in 0.1 (M) phosphate buffer solutions (pH 7.2). The tissues were then fixed in the fixative solutions at room temperature for 20 minutes and then were cut into two pieces after 3 hours of fixation. Total fixation will be for 8-12 hours at 4°C. A change with fresh fixative was given in the middle. Then the tissues were washed with 0.1 (M) phosphate buffer solution (pH 7.2), three times, for one hour duration each at 4°C. The tissues were treated with 1% osmium tetraoxide for 2 hours for post-fixation. After treatment with osmium tetraoxide the tissue pieces were again washed with 0.1 (M) PBS three times. The fixed tissues were then dehydrated with an ascending grade of acetone (30-50-70-80-90-95%) at 4°C and finally kept in dry acetone for 30 minutes at room temperature. After proper dehydration, cleaning was done with toluene twice for 30 minutes followed by infiltration with embedding medium and toluene (1:1). Then embedding was done in pure embedding medium containing araldite (CY212), dodecenyl succinic anhydride (DDSA), 2, 4, 6 tridimethylaminomethyl phenol (DMP-30) and dibutylopthalate. Then polymerization was done by putting the blocks at 50°C for 12 to 14 hours followed by 24 to 48 hours at 60°C. After polymerization 0.5 to 2μm ultra thin sections were cut with ultra microtome. Sections were then placed over copper grids. The sections were stained with uranyl acetate for 10 minutes at dark and then with lead citrate for 10 minutes. The grids were photographed under Transmission electron microscope (Philips CM 10, The Netherlands) at AIIMS, New Delhi.