Chapter 3

MATERIAL AND METHODS
The present endeavour on scanning electron microscopy and life cycle study incorporates two broad aspects on which the methods are scattered in literature and as such a need was felt to present a consolidated form of the methods used during the present study. For the sake of convenience and better understanding, the chapter has been divided into two headings viz.

3.1. Material collection and processing

3.1. A. The host material – fish

In order to get the parasite material, it was important to collect and identify the host material, which was fish forming the final host for this parasite.

3.1. A. 1. Collection of fish

The fish hosts belonging to different fish species were collected alive with the help of fishermen fishing in Dal Lake and River Jhelum. In order to ensure the seasonal prevalence, the specimens were brought fortnightly fresh to the laboratory in large polythene bags containing water. The different seasons were considered as given by (Dar et al., 2002). Spring (March-May), summer (June-August), autumn (September-November), winter (December-February).

The fish hosts were identified using the key given by Kullander (1999).

3.1. A. 2. Observation for parasites

The fish was subjected to a thorough investigation as per the methods employed by Cable (1958) and Mayer and Olsen (1975). The entire outer surface, especially the oral region, the gills, the opercula and the fins were carefully searched for monogenetic flukes and for encysted digenetic trematodes.
(Metacercariae). The gills were then removed in separate petridishes containing normal saline (0.7% NaCl; Cable, 1958).

The hosts were then dissected midventrally and the body cavity scanned for endoparasites. The viscera was taken out and placed in separate petridishes containing normal saline (0.7% NaCl).

The gills of fishes were teased with fine needles in normal saline and examined for parasites. The air bladder was also thoroughly checked for any parasites. The intestines of the fish hosts were fully extended and placed in large petridishes containing normal saline. The intestines were cut into convenient lengths and split open longitudinally.

The worms that usually freed themselves from the mucous by their own active moments were transferred to fresh saline. The anterior end was deeply anchored to the mucosa of the intestine; a few crystals of menthol were added to the normal saline containing the parasites adhered to the intestinal wall. This led to the immobilization of the parasites and loosening of the grip on the intestinal wall and facilitated the detachment of the rostellum from the intestinal wall. The inside of the intestine was scrapped down to the muscle layers. The scrapings and the intestinal contents were mixed with some normal saline and allowed to stand. The upper clearer portion of the liquid was poured off and only the remaining material was examined, first under a magnifying lens and then under a binocular microscope. All worms recovered were counted and transferred to fresh saline.

3. 1. A. 3. Preparation of permanent slides

3. 1. A. 3 a. Preparation for light microscopy

The worms were shaken vigorously in a tube containing 1% NaCl. Cold or lukewarm tap water was also used but care was taken to avoid adverse osmotic effects by restricting the time factor as much as possible. After the mucus had been
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removed, the cestodes were put in distilled water for some minutes prior to killing, which served to relax the specimen's further (Meyer and Olsen, 1975).

Killing and Fixing

The cestodes were fixed on glass slides under a cover glass or slide pressure, while the fixative was run under the cover glass or the slide, depending upon their thickness. The material was then transferred to fresh fixative and kept for half an hour to two hours, depending upon the size (Mayer and Olsen, 1975 and Cable, 1977).

Fixatives

Correct fixation viz. rapid killing and preservation of biological material was utmost importance. The material was fixed to preserve the three dimensional arrangement of constituents of the tissue and of the contents of the cells; it also prevented autolysis and bacterial or fungal attacks and made the tissue resistant to any damage that might be caused by the later procedures. The fixatives used in the present study were:

(i) 10% Formalin

Composition

Formalin (40% Formaldehyde) : 10ml
Distilled water : 90ml

The formalin fixed organisms were washed several times in water 15-30 minutes each to remove all traces of fixative. The material was then processed through 30%, 50%, and 70% alcohol for 30-60 minutes in each. The material was then preserved in 70% alcohol to which 5% glycerin was added.
(ii) Carnoy’s Fixative

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<tr>
<th>Composition</th>
<th>Formula I &amp;</th>
<th>Formula II &amp;</th>
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</thead>
<tbody>
<tr>
<td>Absolute alcohol</td>
<td>60ml</td>
<td>60ml</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>20ml</td>
<td>10ml</td>
</tr>
<tr>
<td>Chloroform</td>
<td>-</td>
<td>30ml</td>
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</table>

Formula I & gave better results than formula II & and hence was used more often. Of all fixatives Carnoy’s fixative was used as a general purpose fixative and gave good results with all materials. The material was then preserved in 70% alcohol.

Staining

To have a differentiation of the biological material, the specimens were stained using the following stains. The stain used in the present study was:

(i) Bullough’s Aceto-alum carmine

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Carmine powder</td>
<td>5gm</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>5ml</td>
</tr>
<tr>
<td>Potash alum</td>
<td>5gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>200ml</td>
</tr>
</tbody>
</table>

Preparation

5gms. of carmine powder, 5ml of glacial acetic acid and 100 ml of distilled water were heated till carmine powder dissolved fully. Then 5gms of potash alum and 100 ml of distilled water were added to the above solution and boiled again. The solution was cooled and filtered. Thymol crystals were added as preservative. The stain was diluted in the ratio of 1:10 or as required.

(ii) Composition of Borax carmine

<table>
<thead>
<tr>
<th>Composition</th>
<th>3gm</th>
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<tbody>
<tr>
<td>Carmine powder</td>
<td>3gm</td>
</tr>
<tr>
<td>Borax</td>
<td>4gm</td>
</tr>
<tr>
<td>Distilled water/ Alcohol (70%)</td>
<td>100ml</td>
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</table>
Borax Carmine, aqueous or alcoholic, used for staining did not give so good results as compared to Aceto-alum carmine. Although both types of staining procedures viz., progressive and regressive were employed, the latter procedure gave better results.

The specimens, preserved in 70-80% alcohol, were hydrated through 50% and 30% alcohol grades, washed twice in distilled water and placed in dilute aceto-alum-carmine for overnight, to get slightly over stained, which helped in controlled differentiation by destaining them in acid water (0.5-1% HCl in distilled water) to the desired intensity. The same procedure was followed with aqueous Borax carmine. However in case of alcoholic Borax carmine, the specimens from the preservatives were washed in 70% alcohol and transferred directly to the diluted stain. The differential destaining was achieved by using acid alcohol (0.5-1% HCl in 70% alcohol).

**Dehydration**

The specimens were passed through an ascending alcohol series i.e., 30%, 50%, 70%, 90%, and 100%. In case of specimens stained in alcoholic stains the series was 70%, 90% and two changes of absolute alcohol. The time required in the alcohols for dehydration depended upon the size of the specimens.

**Dealcoholization**

The specimens were transferred to a dealcoholicizing agent (xylene in the present study) which rendered the specimens transparent and miscible with mountant. The transfer from absolute alcohol to the clearing agent was done gradually to avoid formation of violent diffusion currents which otherwise distort the specimens.
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Mounting

The stained dehydrated and cleared specimens were mounted in Canada Balsam or D.P.X. (Dextrin Plasticized Xylene) mountants. A cover glass was lowered on the specimen. The cover glass was preferably lowered horizontally to avoid displacement of specimens.


For scanning electron microscopy, whole parasites were processed and the steps involved were as follows:

Fixation

The fixation of the material involved the following three steps:

(i) Primary fixation: it was done in glutaraldehyde by keeping the specimens in it for 8hrs at 0-4°C.
(ii) Wash in buffer: The specimens were washed in sodium cacodylate buffer. It was kept overnight in this buffer.
(iii) Post fixation: following the overnight wash in buffer, the material was transferred to a secondary fixative of 1-4% Osmium tetraoxide for post fixation which binds and stabilizes the lipid bilayers and proteins.

Rinse

Following the post fixation, the specimens were then washed in distilled water. The vial containing the material was filled with distilled water and turned upside down once.

Dehydration

The vial containing the material was emptied and filled with 70% alcohol. The vial was turned upside down once, so as to ensure that no water remained in the lid to contaminate or rehydrate the sample. The material was kept in 70%
alcohol for ten minutes. Then the 70% alcohol was removed and replaced immediately with 90% alcohol. Again vial was turned upside down once and kept for ten minutes. Finally the 90% alcohol was replaced with 100% alcohol and the material was kept for ten minutes in it.

Critical point dry

After complete dehydration the material was given a very brief treatment of amyl acetate and then dried at a critical point from liquid CO₂ in a critical point dryer.

Mounting on stubs and plating

This was the final step. In this step the specimens which were to be scanned, were mounted on stubs with silver paste or graphite. The specimen on the stub was then coated with gold or palladium alloy and observed under electron microscope.

3. 1.B. Copepods - The Intermediate Hosts

3. 1. B. 1. Collection and Observation (Fig.9)

1. There are different methods for the collection of copepods and in the present study the collection was done by using a planktonic net having a mesh size of 200μm.

2. A small collection tube was fixed with a simple tag or a rubber band at the bottom of the net.

3. Water, at the collection site, was poured from the top of the net with the help of water sampler. This process was repeated 10-12 times for a single collection tube.

4. The copepods were captured in the collection tube present at the bottom of the net.

5. The netting was removed from the collection tube.
6. The collection tube was covered and labeled.

7. The above processes were repeated till desired quantity of collection was obtained. The collection was taken to laboratory and observed for infection by putting a cover slip on the specimen and observing under microscope.

3. I. B. 2. Techniques for the preparation of copepod slides

3. I. B. 2. a. Initial Treatment of Specimens

**Narcotizing agents**

Narcotizing agents are useful to avoid flexion of the body and to aid retention of egg sacs and gut contents during fixation. The agent is used slowly, drop by drop. The narcotizing agents used in the present study were:

- CO₂ excess: it was produced by bubbling carbon dioxide through water; by pouring soda water into the sample or by adding pieces of dry ice.
- Oxygen-depleted water: the water was boiled for 5-10 minutes and cooled.
- Cold: the specimens were refrigerated in refrigerator, then the fixative was added to the sample at the same temperature.
- Alcohol: pure ethanol was added to the sample.

**Fixation**

The sample bottles were filled ¾ full. Fixation was done within 5-10 minutes after catch. Buffered 4% formalin was used as fixative.

Borax (sodium tetraborate, Na₂B₄O₇) was used as the buffering agent to reduce acidity. To make up a fixative solution, 2g of borax was added to 100ml (40%) formalin and the jar was inverted several times during one hour. This would raise the pH to about 8-8.2. 70-95% ethanol was also used as fixative.
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Staining for sorting
It helps in visual separation of specimen from sediment or detritus-filled samples. In the present study Methylene blue was used for staining. Neutral Red was also be used for staining but did not give good results.

Storage
The specimens were transferred within 7-10 days to ethanol for long term storage because specimens become brittle and setae break off easily when kept in formalin, even buffered for long periods.

70% ethanol and 1% glycerine was used as a long term storage medium to maintain flexibility of specimens. The glycerine prevents evaporation. Methanol and isopropanol are also suitable long term storage media.

3. I. B. 2. b. Microscopic Examination (Phg. 7; Phg. 8)

Pre-treatment to remove soft tissues
The specimens were warmed in 10% KOH for few minutes or they were immersed in a solution of 0.1% household bleach for a few minutes.
The pre-treatment was followed by a brief rinse in distilled water.

Stain
Good stains for copepods are Chlorazol Black E, Fast Green, Acid Fuchsin, Methylene blue. All stains were prepared as 1% solution by weight in distilled water or 70% ethanol. Methylene blue was used often during the present study.

Mounting media
The choice of mounting media depends on the use to be made of the mounted specimen, the type of microscopy employed, and the need for long term preservation. Accordingly there are two types of mounting media:
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i. Temporary mounting media
Glycerine was used as temporary mounting medium. Specimens were brought gradually to isotonic state by transfer or evaporation. The glycerine gave good contrast for microscopic examination, clears specimens, non-toxic, stable almost indefinitely.

ii. Permanent mounting media
There are a number of permanent mounting medium for the copepods. In the present study Glycerine Jellies, polyvinyl lactophenol (PVL), Canada balsam were used as mounting media.

Glycerine jelly

Glycerine jelly is excellent for permanent mounts. They clear only slightly and thus interior structures remain easily visible.

Composition

<table>
<thead>
<tr>
<th>Composition</th>
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<tbody>
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<td>Gelatine</td>
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</tr>
<tr>
<td>Distilled water</td>
<td>60ml</td>
</tr>
<tr>
<td>Glycerine</td>
<td>70ml</td>
</tr>
<tr>
<td>Phenol</td>
<td>1g</td>
</tr>
</tbody>
</table>

Preparation

Gelatin was soaked in distilled water for about two hours. Then glycerine and phenol was added; heated in a water bath (about 40°C or 104°F). stirring gently until the mixture was blended. Then allowed to cool. The temperature was not allowed to rise above 40°C, because the colloid would no longer solidify.

Preparation for glycerine mount: glycerine was added to the alcohol or formalin in which the specimen was kept, until 10% of the storage fluid was glycerine. Then evaporated to 100% glycerine and then transferred to glycerine jelly. A drop of glycerine jelly was placed on a slide and the slide was warmed to 40°C. The whole specimens were added and positioned near the centre of the drop. A cover slip was placed slowly, picking up the edge of the drop of medium. The
mount was allowed to cool and stabilize for at least a few hours before sealing. Transparent finger nail polish was used as the sealant.

Polyvinyl Lactophenol (PVL)

Composition

Polyvinyl alcohol powder 2g
70% acetone 7ml
Distilled water 5ml
Glycerine 5ml
Lactic acid 5ml

2g polyvinyl alcohol powder was placed in 7ml 70% acetone and was stirred. Then Glycerine and Lactic acid were added and mixed well and 5ml distilled water was added. Carefully heated with stirring until mixture was cleared. If too viscous, water was added, few drops at a time. Nail polish was used as the sealant.

Canada Balsam

Canada balsam was also used as the mounting medium. It offers long term preservation.

3. II. Experimental study

For studying the life cycle of cestodes and effect of various factors on the development of cestodes, it was necessary to maintain the hosts under experimental conditions, because under natural condition such parameters were not possible to monitor. For example the effect of temperature on the development of eggs, effect of egg density on proceroid development, etc.
II. A. Maintenance of Copepods under Experimental Conditions (Phg.10)

3. II. A. 1. Rearing copepods

1. For culturing the copepods, collection was done from a standing water source where fish were absent, so that they were free of natural infection. The procedure of collection was same as stated above.

2. The copepods were cultured in small aquaria and plastic containers which were filled with dechlorinated water. Water containing chlorine was dechlorinated by letting it to remain in the open for at least 24 hours or by treating it with chemicals (e.g. Sodium Thiosulfate).

3. Several gravid females were picked up with the help of a small pipette and deposited in each container for starting up the culture. Gravid females were easily recognized because they carry egg sacs externally on both sides of their bodies.

4. The containers containing copepods were placed at the site where they receive the natural light for at least half of the day, but away from direct sun.

5. Copepods were hard to see at the beginning of the set up due to low density. When density increased copepods were seen with the naked eye throughout the containers, swimming and resting.

6. For feeding the egg yolk was used, every 3-4 days.

7. The copepod containers were maintained properly. As any excess feed in the containers decomposed, some would start floating and was removed immediately. This was done by scooping with 800μm sieve and discarding. Sieves were made by cutting a large hole on a large plastic lid and gluing the desired size mesh tightly to cover the hole.
3. II. A. 2. Methodology for the experimental infection to copepods

The gravid tapeworms obtained from the intestine of fish were placed in small petri dishes containing tap water so as to release eggs spontaneously. The hatching of eggs was observed at different temperatures.

For estimating the number of eggs, the methods given by Nie and Kennedy (1993) were followed. Eggs in water in a petri dish were distributed until an even distribution was achieved. The egg density in grid was estimated and eggs were transferred into the experimental dish until the required number was obtained. When a large number of eggs were required, a suspended solution of eggs was prepared by thoroughly shaking them in a sampling tube. The solution was rapidly transferred to a pipette and the number of eggs in the first two drops of the solution was counted. This procedure was repeated few times to check accuracy and then the first two drops were used for the experiment.

Copepods were taken from the cultured containers. Infection of tapeworms was given in petri dishes containing the tapeworm eggs. Procercoid length was measured through the copepod cuticle. Four measurements were taken following the procedure of Dupont and Gabrion (1987): total length of the larva, and three widths respectively at the anterior quarter, the middle and the posterior quarter. The size of the procercoid was then calculated by multiplying the length by the average width. The size of cercomer was not included in the body size. For observing the effect of egg density on the mean number of procercoids, ten petri dishes were setup containing 500, 1000, 2000, 3000, 4000, 6000, 7000, 8000, 10000, 15000, 20000 eggs. Thirty gravid female copepods were added to each petri dish and were counted and examined after 10 days of infection.
3. II. B- Fish Maintenance (Phg.15; Phg. 16 and Phg. 17)

3. II. B. 1. Setting up of an aquarium

Two to three week "conditioning" or "run in" time was given to the water of the new aquarium to get stabilized. Actually it takes time for a healthy population of denitrifying bacteria to develop that can degrade the metabolic waste products of the fish in the aquaria. This is true regardless of the filtration system used. All tanks/aquaria need a biological filtration system.

1. Treating the water to make it toxin free.
   - Water was pre-treated by treating it chemically with sodium thiosulfate (1ml of 1% thiosulfate/10 gallons of water) to remove chlorine.
   - Chlorine was also removed by exposing the water to light for 24 hours, or by aerating it.

2. Location of aquaria was important. It was kept out of a busy section. Too much commotion might frighten the fish initially. Also the aquaria were kept away from direct sun light, in order to avoid algae or heat problem.

3. Large numbers of fish were collected from the region where natural infection of cestodes was mostly absent.

4. About half of the fish were taken randomly and dissected to observe the infection. Due to complete lack of infection in that group of fish, the rest were assumed to be free of infection and maintained in the aquaria.

5. Feed: the golden rule for fish feeding was to give 'little and often'. The amount which was eaten in 15-20 minutes was given. The food was dropped in one corner of the aquaria which helped in developing a feeding routine with the fish. Feed was not used more than once a day and the feed was used early in the day. It was better because they had time to eat the food before dark. This stopped the food from decomposing overnight.
Sometime if the fishes did not take the food and was in excess in the aquaria, it was immediately siphoned out before it scattered.

For feeding the fish, cooked rice and the fish feed was used. The fish feed was made in the laboratory which contained grinded Soya beans, corn and dried fish. All were mixed in specific proportions and water was added to make a smooth paste. Small pallets were formed and were dried to use as fish feed.

Composition of fish feed (Phg.13; Phg. 14 and Phg. 15)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soya beans</td>
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</tr>
<tr>
<td>Fish meal</td>
<td>34g</td>
</tr>
<tr>
<td>Wheat flour</td>
<td>10g</td>
</tr>
<tr>
<td>Vitamins and</td>
<td>2g</td>
</tr>
<tr>
<td>mineral mixture</td>
<td></td>
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</tbody>
</table>

Preparation of feed

Soya beans were grinded to form powder. Similarly the fish meal (dry fry) was grinded. Vitamins and mineral mixture was brought from the market. All the above ingredients were mixed and small quantity of water was added to make a smooth paste. Then pellets of that paste were formed and used as fish feed. For long term preservation, the pellets were dried and kept in an air tight jar.

6. Filtration was done by using simple filters available in market.

7. Temperature. Temperature is a very important part of a fish’s environment. Improper temperatures can adversely affect a fish’s health. High temperatures reduce the amount of oxygen in the water, reducing tank capacity. Thermostators were used to provide required temperatures. Temperature was monitored using a thermometer.

8. pH: It was maintained in the range of 6.8-7.2. Higher pH can enhance the formation of ammonia in water, while low pH can adversely affect the
Phgs. 7-8. Copepod intermediate host of *B. acheilognathi*

Phg. 9. Planktonic net

Phg. 10. Maintenance of copepod under experimental conditions

Phg. 11. Maintenance of *Tubifex* under experimental conditions

Phg. 12. Dry fry

Phg. 13. Soya beans

Phg. 14. Wheat flour

Phgs. 12-14. Different constituents of Fish feed
Phgs. 15-17. Maintenance of Fish-final host under experimental condition
function of the fish gills and can be detrimental to the growth of denitrifying bacteria. Water pH was adjusted with commercially available buffers. Water pH was measured in tanks using commercial test kits.

9. Oxygen: like all animals, oxygen is essential for the survival of fish. Oxygen is absorbed at the water-air interface. Turbulence was provided to the water through the use of a pump or an air supply, which increased the oxygenation of the water. Oxygen concentration was calculated by using commercial kit. It was kept between 5 and 7 ppm.

10. About 70% water was changed each day by siphoning to remove toxins and other wastes.

11. Aquarium lids and net was used for the protection of fish.

3. II. B. 2. Experimental Infection to fish

1. Fish were starved 24hrs before given infection of the cestode parasites, and were kept in small aquaria with infected copepods containing the larval stage of the cestode.

2. Fish were killed 15, 35, 110 and 120 days post infection (DPI) and observed for the infection.

Microscopy and Micrometry

Light Microscopy

Light microscopy was conducted under Olympus Research microscope, (DP 12, 4D 0082, U TVO 35 X C), with lens combination of 7x, 10x and 15x eye pieces and 4x, 10x, 40x, and 100x objectives. Photographs were taken with Olympus digital image analyser. The drawings for identification was made with the help of prism type camera lucida.
**Material and Methods**

**Micrometry**

The measurements were taken with help of:

(a) Objective (stage) micrometer: by drawing the scale of the objective micrometer under different lens combinations and then taking measurements directly from the drawings drawn under the same magnification.

(b) Objective and ocular micrometers: by standardising the ocular micrometer with the objective (stage) micrometer and later measuring the parasites or parts with the help of Ocular micrometer only.

**Data analysis**

Correlation analysis and Chi square test were employed to calculate the relationship between different parameters and their significance at $P < 0.05$. Statistical package MINITAB 2002 v13.2 was used to analyse the data. The following formulae as given by Morgolis *et al.* (1982) were adopted to calculate the Prevalence, Mean intensity and Abundance.

\[
\text{Prevalence} = \frac{\text{Total number of hosts infected}}{\text{Total number of hosts examined}} \times 100
\]

\[
\text{Mean intensity} = \frac{\text{Total number of parasites}}{\text{Total number of hosts infected}}
\]

\[
\text{Abundance} = \frac{\text{Total number of parasites}}{\text{Total number of hosts infected}}
\]