

3.1.1 General Procedures for Parasitology

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A. Introduction

The examination of fish for parasites may be required as part of a routine monitoring program for all disease agents including bacterial and virological agents, as part of a routine monitoring program for inspection of parasites only, or as a diagnostic search for a specific disease/mortality causing agent.

An external parasitological examination can be the first part of a routine monitoring program or diagnostic process. When an internal parasitological examination is required, it should follow the bacteriological examination, which requires aseptic conditions. Where multiple fish samples are required for bacteriology, virology, histology, and parasitology, the sampling methods and order of process will be dictated by the number of fish samples available. Where numbers are not restrictive, the sample size may be increased accordingly and bacteriology samples taken from fish selected for this process only. Tissues for virology, parasitology, and/or histology may be taken from remaining fish.

In this chapter, suggested methodologies are presented to facilitate the recovery, storage, and shipping of parasite specimens. Only those parasites that are currently recognized as being responsible for the majority of "disease" situations in fish culture are individually discussed. A simple key to the major taxa of adult parasites of fish is also provided in Section 1, 3.1.2 A Key to Major Taxa of Adult Parasites of Fishes.

The specific parasites included in this section were determined by discussion with fish pathologists and aquaculturists throughout North America from the information provided from the Blue Book questionnaire (AFS Newsletter, Fish Health Section Volume 17, 1989). Additional parasites will be added as deemed necessary.

B. Sample Submission

The proper recovery and identification of fish parasites is dependent upon the state of freshness of the host. Thus, live fish samples are always preferred. If this is not possible, the usual order of preference for the submission of samples is: (i) freshly killed and packed on ice, (ii) preserved in an appropriate fixative, and (iii) deep frozen. The sediment in each container should be examined for external parasites that have fallen off or internal parasites that have been regurgitated or exited via the anus.

The survival of parasites following the host's death will be dependent upon the temperature at which the fish is maintained. The colder (i.e. on ice, not frozen) the host is kept, the better the survival of the parasites. Certain parasites may exhibit morphological changes following the death of the host. In addition, internal parasites may migrate from their sites of infection and external parasites may detach from their dead host. Whatever the process by which the fish host samples are submitted to a diagnostic lab, the host should be prepared as soon as is possible following its death.

A guide to methods for the packing and transport of fish samples is given by Mitchell and Hoffman (1985). The examination of whole, preserved fish is sometimes difficult due to the rigidity of the fixed muscle. Under certain conditions, where examination of fresh material is not practical or required, the viscera may be removed *in toto* by cutting at the pharyngeal/esophageal junction and at the anus. The swim bladder and kidney are also removed at this time. The viscera are then fixed and stored until examined. Once fixed, the viscera may also be shipped by wrapping them in a 5 to 10% formalin-soaked paper towel inside a sealed plastic bag. The two ends at the esophagus and anus can each be tied with a piece of thread to prevent any loss of their contents.

C. General Necropsy Procedures

The following is a general procedure for the examination of a fish for parasites. Where numbers of fish, time, or facilities are limiting, this procedure may be adapted to suit a particular situation. Specific methods for the recovery and treatment of specific parasites are given within the individual chapters.

1. Examination of Blood

If an examination for blood parasites is required, the sample must be collected from freshly killed fish before the blood clots and/or hemolysis occurs.

Blood may be collected via any of the following methods and host sites: (i) cardiac puncture with a syringe or sharpened microhematocrit tube as described by Goede (1989), (ii) entering the dorsal aorta via the oral cavity or along the lateral line, (iii) from the caudal vein by syringe or by severing the caudal peduncle, or (iv) by cutting a gill vessel.

Regardless of the route and method used, the collecting vessel should be heparinized to prevent clotting. The collecting syringe need not be heparinized if the blood is transferred immediately to the collecting vessel.

Motile living parasites (e.g. flagellates and larval nematodes) may be examined in a fresh drop of blood diluted with physiological saline (8.5 gm NaCl/L of distilled water). Flagellates are easily recovered from the white cell or "buffy" layer of the blood sample after microhematocrit

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centrifugation. Fresh blood should be mixed with an equal or excess amount of saline to facilitate examination.

A blood smear may be prepared for later examination. A single drop of blood is required. This is easily achieved by touching a blood filled microhematocrit tube to one end of a glass slide. A second glass slide is then drawn to the edge of the drop, and pulled quickly but smoothly, creating a film, one cell layer thick (Figure 1). The smear is air dried, fixed for 10 minutes in methanol, and stained with a Romanovsky stain (Humason 1979).

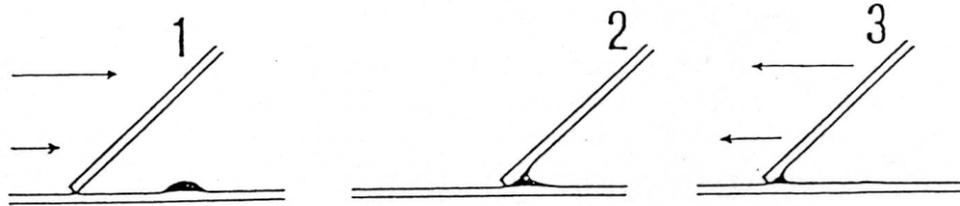


Figure 1. Preparation of a blood smear (from Moller and Anders, 1986).

2. External Examination

The entire surface of the fish should be thoroughly examined including the oral and opercular cavities, the nares, gills, and fins. If there is a time restraint or a large number of fish are to be inspected, most external parasites can be detected by examining a mucous scraping from a number of sites along the body, including the head, pectoral and caudal fins. The external surface of small fish (i.e. fingerlings) may be examined with the aid of a dissecting microscope, whereas the use of a hand-held lens can be useful in examining the surface of larger fish.

Protozoans and monogenetic trematodes may be removed from the external surfaces and gills by placing the fish or the individual tissues in a solution such as 1:4000 formalin. Small parasitic specimens are easily handled with a pasteur pipette. The nares may be examined by flushing with saline in a pasteur pipette and a wet mount or smear made of the wash fluid.

A mucous smear can be made by touching a glass or cover slip onto the side of the fish or mucus may also be collected with the non-cutting edge of a scalpel and wiped onto a slide. A fresh preparation may be mixed with saline and examined with a compound microscope, using a 10 to 40X objective. The use of phase contrast may be helpful for the recognition of protozoans. When preparing a fresh smear, care must be taken when placing the cover slip onto the material. Artifacts can result from excessive pressure. If necessary, tap water may be substituted for saline in any of the preparations given in these procedures. However, the use of saline may be critical to maintain a suitable osmotic environment for certain parasites, and is the medium of choice.

If there is enough saline on the slide and the cover slip is lowered properly, no air bubbles should be present. Excess saline can be removed by touching a paper towel to one edge of the cover slip. In the event there is insufficient saline, it may be added at the edge of the cover slip.

If protozoan cysts or encysted helminths are observed upon examination, pressure may be gently applied to the cover slip to free the organisms. Encysted worms should be freed before fixing.

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a. Opercula and Gills

The opercles are cut off, placed into a petri dish, and examined under a dissecting microscope. The gill arches are removed separately and placed into a petri dish filled with saline and examined with a dissecting microscope. A small number of gill filaments can be more closely examined with a compound microscope. If the fish is small enough, an entire gill arch can be placed on the slide in saline and covered with a cover slip. Otherwise, a section of gill arch is cut out, placed onto the slide, the filaments cut off with a scalpel, and the cartilaginous arch removed. If the fish is very large, some filament tips can be removed with scissors and placed directly onto a drop of saline on a slide. If desired, gill preparations can be minced on the slide with a scalpel to facilitate parasite detection. A drop or two of 1% methylene blue or 1% methyl green nuclear stain onto a wet mount will facilitate protozoan detection. These methodologies can also be applied to fin and mucous preparations.

b. Eyes

The eyes should first be examined while still in the orbits. Each eye can be removed using forceps and scissors and placed into a small petri dish with enough saline to cover the eye. The eye is cut open and the lens, humor, and retina examined. If the lens is removed, the remaining contents of the eye can be prepared as a wet mount and examined with a compound microscope.

3. Internal Examination

If a bacteriological examination is to be done, in addition to a parasitic examination, the external surface of the fish should be disinfected before exposing the body cavity.

The fish is laid on one side and is opened from the mouth to the anal region. This is accomplished by a ventral incision. A pair of blunt/sharp scissors should be used, with the blunt end going into the body cavity to minimize damage to the internal organs. The lateral musculature may be removed (Post 1987), but this is an individual preference.

a. Abdominal Cavity

The body cavity is examined first for encysted parasites. The heart is then removed and placed into a petri dish filled with saline. The viscera are removed *in toto*, by cutting the pharyngeal/esophageal junction and the large intestine at the anus. The viscera are placed into a saline filled petri dish. If a detailed examination is required, the liver, gall bladder, spleen, esophagus, stomach, pyloric caeca/pancreas, and intestine (which may be sub-divided as required) are removed and placed into individual petri dishes for examination. The swim bladder is then removed and similarly examined. Care should be taken not to deflate the swim bladder as the initial examination is facilitated when it is full. Removal and examination of the kidneys, ureters, and urinary bladder is now possible.

Smears of the ureter, gall bladder, and urinary bladder contents may be made by simply teasing these organs apart on a slide or utilizing a tuberculin needle and syringe to remove the contents of the latter two. Squashes may also be prepared of these organs as well as the kidney, liver, spleen, and gonads.

Cysts should be removed, placed onto a microscope slide, and the cyst wall teased apart to release the contents. If this is not possible, a cover slip may be placed on top of the cyst and sufficient pressure applied to squash the cyst. Encysted helminths should be freed before fixing.

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The alimentary canal segments are stretched out in the petri dish and cut open longitudinally and the contents examined. Smears (wet and fixed) of the intestinal epithelium and contents should be made and examined for the presence of parasites. The alimentary tract of larger fish may be flushed through with saline and the gut contents examined separately from the tract wall.

A representative number of pyloric caeca can be examined by expressing their contents. This is easily done by cutting caeca off at their intestinal end and grabbing their blunt terminal end with a pair of forceps. A second pair of forceps is used to run down the individual caecum, from the blunt end to the open end and squeezing out the contents. It is often not practical to cut open large numbers of caeca, unless a quantitative study is required.

b. Brain

The brain of the fish is usually the last organ to be removed as this is the most difficult to access. It is not necessary that the brain remain totally intact for a parasitological examination. For fish less than 15 cm in length, the brain case may be cut longitudinally with the use of a pair of blunt/sharp scissors. The blunt end is placed in the mouth and the pointed end is lined up directly between the eyes. A single cut will usually expose the brain, which can be removed with forceps, squashed, and examined. In the case of larger fish, a pair of bone cutters may be required.

c. Musculature

The musculature is usually examined last by: (a) slicing the epaxial musculature at regular intervals, or (b) removing the two fillets, slicing, and/or candling them.

Candling is the use of transmitted light through a fish fillet to aid in the illumination and detection of parasite stages within the musculature (pp 338-339, Moller and Anders 1983). The efficiency of this method is dependent upon the thickness of the fillet. Squashes may also be necessary for the detection of cysts. Digestion with 0.5% pepsin powder in water and 0.5% HCl at 37°C may facilitate quantitative studies (pp 7-8, Hoffman 1967).

D. Fixation and Treatment of Parasites

There are a number of fixatives that are useful for the preservation and storage of fish parasites. These include formol-alcohol (i.e. AFA), Bouin's, and formalin (Humason 1979). Formalin is the most common fixative used, but there is confusion with regards to its use.

The formalin product purchased directly from the manufacturers is a saturated aqueous solution containing between 37 and 40% formaldehyde gas, by weight. This solution is a 100% stock solution of formalin. To make a 10% solution of formalin for preserving samples, it is necessary to dilute 1 part of the 100% stock solution with 9 parts of water. A 5% solution will require that 1 part of the 100% stock solution be diluted with 19 parts of water. These solutions may be buffered.

Certain literature may refer to the use of a 4% formaldehyde solution (Roberts 1989; Moller and Anders 1986). This is a 10% aqueous solution of the commercial stock formalin, which contains 40% by weight formaldehyde gas. In other words, it is calculated based upon the stock formaldehyde gas concentration (i.e. 10% x 40% of stock = 4%).

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1. Protozoan Smears/Small Helminths

If a temporary preparation is required for protozoan smears or small helminths, this may be done in one of two ways. A preparation may be stored overnight as follows: a dampened paper towel is placed in the bottom of a petri dish, the specimen slide placed on top of the paper towel, the lid placed on the dish, and then put into a 4°C refrigerator. A sample preparation may be retained for several days by adding a ring of nail polish or petroleum jelly around the perimeter of the cover slip to prevent dehydration and keeping this under refrigeration.

If any temporary preparation is to be fixed, the fixative can be added to one side of the cover slip. A dry paper towel is touched to the opposite side of the cover slip. This will cause the saline under the cover slip to be drawn into the paper and create a capillary action under the cover slip, which will draw the fixative through the sample.

2. Helminths, Acanthocephalans, and Leeches

When necessary, living trematodes, cestodes, and leeches should be fixed under light pressure of a cover slip or glass slide. The saline is replaced slowly with warm fixative.

All helminth parasites should be cleaned with saline before fixation. This is accomplished by placing the parasite into an appropriate sized vial filled with saline, capping it and shaking it vigorously. Small parasites are best handled by sucking them up with a pasteur pipette to minimize damage, whereas larger parasites can be handled with forceps. The fluid is decanted and the procedure repeated as necessary. (Note: decant into a jar, not over a drain.)

Monogeneans, trematodes, cestodes, acanthocephalans, and leeches can be fixed in 5-10% neutral buffered formalin for 24 hours and transferred to 70% ethanol for storage and/or shipping. Many helminth parasites may be relaxed in fresh water before fixation. Some parasitologists suggest relaxing cestodes in 80°C tap water and then slowly replacing the water with fixative. Water or fixative that is too hot may result in blisters to the tegument or cuticle of certain parasites, therefore, the use of warm (ca. 50°C) formalin to relax and fix live parasites is recommended.

Live acanthocephala should be left in distilled water overnight in the refrigerator to induce eversion of the proboscis before fixation in warm formalin. Large nematodes and acanthocephalans may have to have holes poked in them with fine dissecting needles. Entomology pins mounted onto the end of small wooden dowels are excellent for this purpose as well as for manipulating small, delicate parasites. Live nematodes and crustaceans are fixed for 24 hours in warm solution of 5 to 10% glycerin in 70% ethanol and transferred to fresh solution for storage. These may also be fixed in 10% formalin then transferred to glycerin/ethanol for storage.

Make sure that all containers, regardless of their contents, are properly labeled. Labels should be written in pencil and the label placed inside the container. A second label can be put on the outside, but the inside is most important.

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