



SECTION 1

SAMPLING FOR DISEASE DIAGNOSIS

The ideal specimens for disease investigation or health monitoring are live fish. Samples can be taken from these either on site or transported live to the appropriate laboratory.

Transport of live fish

Place the fish, representative of the problem, in a plastic bag filled to approximately a third with water and two thirds oxygen. Seal the bag with cable ties or equivalent and place in another bag and seal again. Then place the bag on ice or cool-packs in an insulated box e.g. polystyrene, place more ice on top and seal the container.

The maximum transport time depends on water temperature, and the ratio between biomass, water volume and oxygen. As a rough guide the transport time should not exceed 12 hours and the biomass should not exceed one third of the water volume. Transport time is significantly reduced if oxygen is not used.

Live fish weighing more than approximately 300g should not be sent by normal goods transport (air, rail or road), but should either be sampled on site or sent via specialised forms of transport.

Transport of fresh material

Unopened fish, reproductive products, virology samples, fish heads (for *Myxobolus*) may be dispatched for laboratory investigation in the fresh state.

All samples must be chilled to as close to 0°C with out freezing. Pack samples in ice and in an insulated container and dispatch. The maximum transport time is 24 hours.

PARASITOLOGICAL SAMPLING OF FISH

- a) Examination of skin: first stun the fish with a sharp blow to the head. Then take scrapings for microscopic examination using a scalpel and scrape from front to back of the fish or around the fins (Figure A). Place scraping on a clean glass slide with a drop of water from the holding facility and cover with a coverslip.
- b) Examination of gills: following gross examination of the gills, clip a small portion of gill lamellae with sharp scissors and place on glass slide (alternatively scrap the gill lamellae with a scalpel), add a drop of the holding tank water and cover with a coverslip. Examine under low power with high contrast or phase.
- c) Examination of other organs: any other organs suspected of having parasitic infection can have squash preparations made from small sub-samples of tissue and examined similarly using light microscopy.

- d) Record and/or draw your findings.



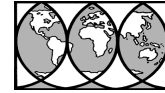
Figure A. Skin scrape of salmon parr using a scalpel.

HISTOLOGICAL SAMPLING OF FISH

Histology encompasses the scientific area concerned with the structure of tissues and histopathology the relevant branch of pathology. Histopathology can therefore provide information on the processes and changes occurring in tissues and in many cases form the basis for disease diagnosis and prognosis.

Accurate sampling of tissues for histology is a vital part in the diagnostic procedure and to follow are guidelines for on site sampling. Before sampling any fish note any behavioural abnormalities or visible external lesions.

- 1) As with other diagnostic procedures, a mixture of sick (moribund) and healthy fish should be sampled. Dead fish (recent mortalities) provide little accurate information and are virtually useless for histology.
- 2) Sacrifice the chosen fish by stunning the fish by a blow to the head or through anaesthesia.
- 3) Phosphate buffered formalin is the tissue fixative of choice for the majority of samples. 10% formalin is the usual concentration (formalin being 40% formaldehyde). Care should be taken as formalin is an irritant, especially to eyes.
- 4) Following killing of the fish, samples should be immersed in formalin as quickly as possible (all tissues should be sampled within five minutes of killing) as post-mortem changes will occur rapidly in these cold blooded animals.



- 5) Remove a small piece of tissue (<math><1\text{cm}^2</math>) from the organs and include any areas showing gross abnormalities.
- 6) Sample gill, heart, spleen, liver, pyloric caecae/pancreas, kidney, section of skin and muscle (at lateral line) and brain. Small fish or fry may be sampled whole with the abdomen opened to allow proper fixation of the internal organs.
- 7) Ensure amount of formalin in pot is approximately 10 times the amount of tissue.
- 8) Record any details about fish on paper e.g. body condition, weight, length, feeding, internal appearance, etc...
- 9) Label the sampling pot with a water proof marker and ensure that each pot will not leak.
- 10) Dispatch/deliver samples with relevant details and clinical history and contact the diagnostic service by telephone or fax.

Phosphate Buffered Formalin Solution (PBFS)

Formula:	Formaldehyde (40%)	100ml
	Water (tap OK if distilled unavailable)	900ml
	disodium hydrogen orthophosphate	6g
	sodium dihydrogen orthophosphate	5.5g

Dissolve dry powder in water, and then add formaldehyde.

THE GRAM STAIN

This is a differential staining method and is the first characterisation test applied to all bacterial isolates. It demonstrates bacteria of different types:

Gram positive - those which resist decolourisation
 - stain blue/purple

Gram negative - those which are decolourised
 - stain red/pink

The difference in colour reaction is due to the different chemical composition of the cell wall and membrane. This stain will also reveal the general shape of the bacteria.

It can be useful in the diagnosis of Bacterial Kidney Disease (BKD) and rainbow trout fry syndrome (RTFS) using kidney material taken directly from the fish.



METHOD

1. Take a clean slide and using a sterile loop, emulsify a small amount of material in a drop of sterile saline or distilled water.
2. Allow the slide to air dry, then heat fix it by passing it through a flame three times. Allow slide to cool.
3. Flood the slide with crystal violet for one minute.
4. Holding the slide at a steep angle, wash off the stain with Grams iodine and allow to sit for one minute.
5. Tip off the iodine and pour the alcohol/acetone mixture over the slide from the upper end, so as to cover its whole surface. Repeat until no more colour runs off, then wash gently in water.
6. After shaking off the excess water, flood the slide with the safranin counterstain and leave for approximately two minutes.
7. Wash the slide in water and dry.
8. Examine under X40 objective, then under oil immersion with X100 objective.

BACTERIOLOGICAL SAMPLING OF FISH

1) Examination and direct inoculation of solid media

Before examining a fish internally, the external body surface, including gills, tail and fins should be examined for the presence of any lesions. Observations should always be recorded on paper. Samples from these sites can be taken by searing the surface with a hot scalpel blade followed by insertion of a sterile bacteriological loop or swab. Material from the loop/swab is then plated out onto suitable agar medium by the spread plate technique.

Once external examination or sampling has been carried out, the body surface is opened to expose the internal organs. Care must be taken not to puncture the gastrointestinal tract. In the absence of any visible internal lesions a sample of kidney is taken and inoculated onto suitable agar medium. The surface of the kidney (or other organ) should be seared with a hot scalpel blade before insertion of the sterile loop (Figure B).

Agar plates containing the streaked out samples should be incubated and examined daily for any evidence of growth. The majority of bacterial fish pathogens will grow on Tryptone Soya Agar (TSA) within seven days. Media such as Marine Agar or TSA plus

1.5% salt (NaCl) can be used for marine pathogens.



Figure B. Bacteriological sampling of salmon kidney using a hot wire loop.

2) Kidney smears

Direct examination of Gram stained kidney smears may give an indication of bacterial septicaemia and is especially useful in the examination of fish for Bacterial Kidney Disease (BKD).

A small portion of kidney is emulsified in a loopful of sterile physiological saline on a microscope slide, allowed to air dry then fixed by passing the slide through a bunsen flame several times.

Direct observation of the slide is carried out using the X40 and X100 (oil immersion) objectives after being stained using Gram's method.

3) Bacteriology plates or swabs can be dispatched to the lab for investigation but must be clearly identified and labelled. A written note regarding the history and clinical appearance of the fish and samples taken is important.

INOCULATION OF AN AGAR PLATE

Petri dishes containing solid medium (agar) are used to provide a large surface of media for the cultivation of micro-organisms. Inoculation of an agar plate is often carried out using the **streak plate technique**. This involves diluting the culture or other sample, e.g. kidney material, by smearing it across the surface of the agar. Organisms present in the sample will be separated and after suitable incubation each organism present will give rise to a colony. Although this colony contains many millions of organisms they will have all originated from one, and therefore all organisms in one colony are identical.

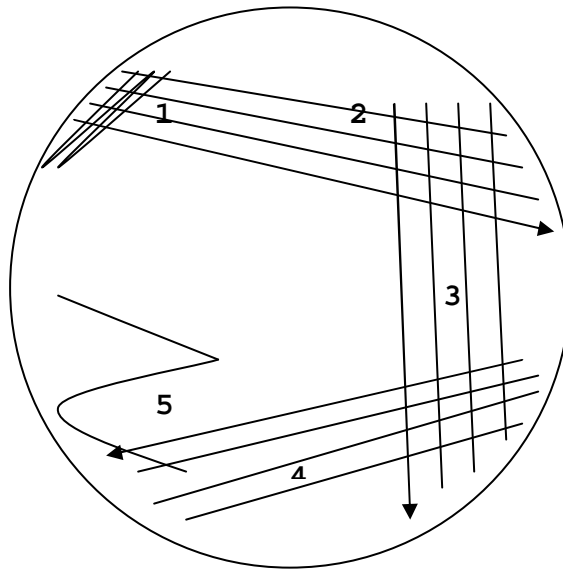
By using this method organisms can be cultured in the laboratory and if a mixed culture is present it will become apparent on plating out. This is essential before starting



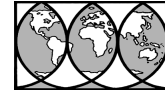
identification procedures as methods are only valid when carried out on pure cultures, i.e. cultures containing one type of organism.

Care should be taken that plates used for this purpose are dry. Also avoid unnecessary exposure of the agar surface to potential contamination from the environment throughout the procedure.

STREAK PLATE TECHNIQUE



1. Sterilise a bacteriological loop and allow to cool.
2. Pick up a small amount of sample using the sterile loop.
3. Inoculate the sample on a segment of the surface of the culture medium (1).
4. Sterilise the loop, allow to cool and touch edge of uninoculated area of medium to ensure coolness.
5. Spread part of the sample over about a quarter of the plate by making 3-4 parallel streaks with the loop (2).
6. Repeat streaking procedure as shown (3,4 & 5), sterilising and cooling the loop between each sequence.
7. Label the underside (not lid) of the plate, writing only at the edge, and incubate or dispatch with related clinical notes and history. Ensure dispatched samples are



adequately padded to protect against physical damage.

BLOOD SAMPLING

Fish blood may be sampled for disease monitoring or health status analyses in the following areas:

- i) haematology (examination of blood cells and blood cell indices), e.g. red blood cell count, haematocrit
- ii) blood biochemistry e.g. hormones, enzymes, etc.
- iii) plasma parameters e.g. plasma chlorides (for salt water challenge tests in salmon)
- iv) serology for pathogen antibodies i.e. salmon pancreas disease virus antibody screening
- v) virology and bacteriology: some micro-organisms can be screened for using frank blood.

For biochemistry and haematology an anticoagulant should be used in the blood collection tube and this is normally heparin (heparinised tubes can be purchased), however, the laboratory where samples are being submitted should be consulted for their normal requirements. For serology, bacteriology and virology blood should be taken without anticoagulants.

Where fish are large enough, blood samples can be taken under anaesthesia i.e. non-lethal sampling. The normal sampling site is from the tail vein and the easiest approach is ventrally at midline towards the vertebral column.

VIROLOGICAL SAMPLING

For virological sampling the fish organs and transport medium/conditions will be determined by the virus/es suspected. The laboratory where samples will be submitted should be contacted prior to sampling.

Further reading

Collins, R. (1993) Principles of Disease Diagnosis. In: **Aquaculture for veterinarians; fish husbandry and medicine**. Edited by L. Brown, Pergamon Press, Oxford.

O.I.E. (2003) **Manual of Diagnostic Tests for Aquatic Animals**, 4th edition. O.I.E., Paris, France.