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Laboratory approaches towards disease diagnosis: Histopathology & Parasitology

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Successful diagnosis is the most important step in any disease control programme, the outcome of which determines the ultimate success or failure of the programme. Once an infection or disease is suspected, the next step is to draw a diagnostic procedure, to fix the root cause of the problem.

When and where to start an investigation?

In farm conditions,

- Any mortality greater than 0.3% and less than 0.5% per day should be suspected and investigated.
- Mortality greater than 0.5% but less than 1.5% indicates that a fish health problem/disease is present.
- Mortality reaching/exceeding 1.5% per day should be treated as an epizootic
- The sick animals should be isolated as much as possible to prevent transmission of the disease to other lots and control measures initiated.

Sampling procedures

In clinical cases of disease (0.5% mortality/day) 10 moribund fish or shellfish are generally a sufficient sample size to make a diagnosis. In situations where no excessive mortality or clinical disease is apparent, a larger sample size of 60 animals may be necessary. However, depending upon individual circumstances, sample sizes may vary between 10 and 60.

The diagnostic procedure may include a single diagnostic test or a combination of tests. In the case of routine pathogen watch or health monitoring, a set of selected diagnostic tests are performed to cover the potential pathogens. The approach generally followed is location specific and problem specific, where the first consideration is the availability of the diagnostic facility and expertise. There is no hard and fast method, which can be applied for all cases.

Records/History on	Water source and Pond, Water parametersStocking, feeding, growth performance, handling, Recent pathologies and treatments EpizootiologyThe more precise and consistent the farm records are the more likely it is for the pathologist to reach a sound diagnosis
On site observation of	Water conditions / soil / benthos and plankton samples. Live / sick fish for shoaling behaviour, reactions to stimuli and feeding. Presence of dead

	and moribund fish. External lesions (ulcers, exophthalmus, reddening, fin erosion)
Examination	Gross external examination for parasitesPreparation of smears
Sampling for	Laboratory examination (whole fish, organs) for Bacteriological, fungal examinations Blood tests / Rapid diagnostic testsElectron Microscopy
On site necropsy	Skin, fins, gills, internal organs Sampling for histopathology / virology
Laboratory analysis	Bacteriology, Parasitolgy, Histopatholgy, Serology, PCR / DNA Probes

Importance of documentation/case sheet

Majority of disease problems in fish are linked intimately to water quality and management issues, factors that do not always translate into directly observable pathology. Interpreting the cause of disease based solely on biopsies or necropsy may be misleading. History of disease at the facility or in the region, farm design, source of seed stock, type of feed used, environmental conditions etc. should be recorded. This systematic record keeping will help to arrive at a primary diagnosis but need not be conclusive. Behavioural aspects like movement pattern, feeding pattern, morbidity etc. along with gross clinical signs such as lesions, haemorrhage, colour changes, fouling etc should also be recorded.

Transportation of samples to the lab

As far as possible live fish are preferred and samples should be immediately send after removal from water. If dead, they should be held on ice and processed within 1-2 hrs. Bacteriological samples should be taken first to reduce chances of contamination and virology samples should be processed within 48 hrs.

Laboratory work sheet: A laboratory case/work sheet has to be kept with all the details of the investigation including the case history (See appendix I & II)

Standard Necropsy Procedures for Finfish (adapted from Theodore R. Meyers)

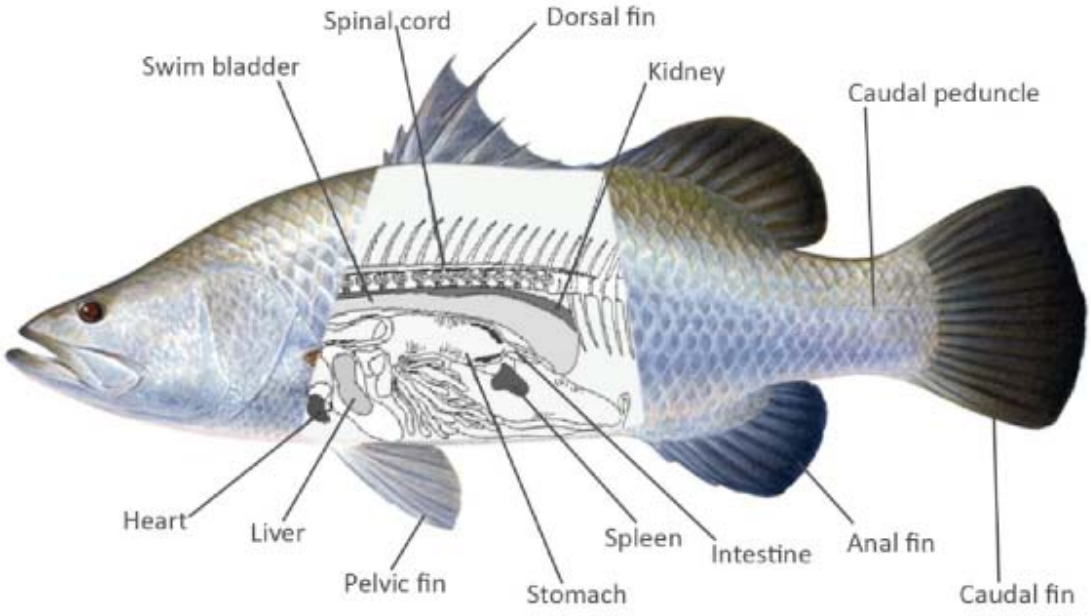
As a first step the sample is given an accession number and the case data information received with the sample should be used to fill out the laboratory worksheet. Live fish should be examined for behavioral abnormalities (spiral swimming, flashing, flared gill opercula, prostration, etc.) then anesthetized to avoid tissue artifacts caused by alternate methods of euthanasia such as pithing or a blow to the head. Some external abnormalities (whitened or eroded fin tips, cloudy cornea, body discoloration, excessive mucus) are best observed while the fish is submerged in water. In many cases postmortem changes in fish received dead will prevent this latter opportunity.

General Necropsy Procedure

- The fish should first be examined for external abnormalities or lesions that could include: poor body condition; exophthalmia; cloudy cornea or lens opacity; hemorrhaging within the anterior chamber of the eyes, fins, body surface or body orifices (anus, nares, mouth, gill chamber), frayed or missing fins; gas bubbles within the fin rays or connective tissues of the eyes; ulcerations, abscesses, abrasions; body discolorations; excessive mucus; trailing fecal casts or rectal prolapse; external foreign bodies such as fungus, metazoan or protozoal parasites,

cysts or tissue growths; potbelly or other protrusion or body malformations (spinal deformities, cranial swelling, shortened opercula, pugheadedness, microeye).

- External lesions such as ulcerations or abrasions should be struck onto TSA (Tryptone Soy Agar) for bacteriological studies. Use of TSA with 1% NaCl may be necessary depending upon case information and whether fish are in saltwater and a halophilic bacterial pathogen is suspected.
- A peripheral blood smear should be made by excising the caudal peduncle (for small fish) and allowing a drop of blood to be deposited near the frosted end of a clean glass slide. The blood is smeared before clotting with a second glass slide by touching the drop with the slide at a 45^{o} angle to the first slide and pushing the angled slide to the end of the first slide. Capillarity draws the smear across the first slide and the smaller the angle the thicker the smear. Stain the smears using Giemsa and observe under the microscope at 1000X. Larger fish may be bled by caudal vein puncture.



- Fish should be placed on their right sides for performance of the remaining necropsy procedures. Skin scrapes of normal and lesion areas mounted with a drop of PBS (Phosphate Buffered Saline) and coverslip on a glass slide should be made by using either the edge of the coverslip as the scraping instrument, or a scalpel. Bacteria or fungus from lesion areas or protozoal parasites such as *Ichthyobodo* and *Trichodina* are common subjects to look for beginning at 40x and then at 200X on a compound microscope.
- Wet mounts of gill filaments are made by using a small pair of surgical scissors to remove a portion of one gill arch. Gill filaments should be slightly teased apart for good viewing of filament and lamellar profiles and mounted in PBS with or without a coverslip. These should be examined immediately since branchial epithelium rapidly deteriorates causing postmortem artifact. Look

for gas bubbles in the capillaries, telangiectasia, hyperplasia, external parasites (bacterial, protozoal, fungal, metazoan), or other foreign bodies.

- Disinfect the outer surface of the fish by flooding with 70% ethanol. Disinfect a pair of scissors, forceps and scalpel by immersion in 100% ethanol and passing the instruments through a Bunsen flame allowing the alcohol to ignite and burn off. Repeat one or two more times. Wipe instruments clean of any organic matter beforehand for effective disinfection.
- The abdominal cavity is entered by pulling the pectoral fin with sterile forceps while cutting into the abdominal wall at the base of the pectoral fin with a pair of small sterile scissors. The cut is continued dorsally to just below the lateral line where resistance is encountered. Start again at the base of the pectoral fin and continue the incision towards the posterior of the fish along the ventral abdominal wall to the vent. Stay slightly above the intestinal tract when making the incision so that it is not punctured, thereby contaminating the tissues. At the vent continue dorsally to just below the lateral line and continue cutting anteriorly to connect with the first incision. Remove the flap of abdominal tissue, thus exposing the internal viscera and cavity. When done correctly on a moribund specimen the air bladder should remain inflated and the GI tract completely intact. Instruments may need wiping of organic material and flaming repeatedly during this procedure.
- Visually examine viscera (heart, liver and gall bladder, kidney, pancreas, adipose tissue, spleen, air bladder, pyloric caeca and entire GI tract) for abnormalities such as: discoloration or mottled appearance; enlargement (hypertrophy); hemorrhage or erythema; abscesses or cysts; fluid in the abdominal cavity (ascites causing potbelly); foreign bodies such as fungus, metazoan parasites or tissue growths, etc.
- If bacteriologic samples are to be taken they should be struck onto TSA from the kidney and/or from visceral lesions before other samples are taken to avoid bacterial contamination.
- Tissues to be taken for viral assay of larger fish (kidney/spleen pool) should also be placed into sterile tissue culture fluid for refrigeration and homogenization at a later time. Fry are generally processed whole for virology
- If the spleen has not been completely removed for virus assay, a spleen squash can be made by placing a cut section of the tissue with a drop of PBS on a glass slide and covering with a coverslip. Whole spleen squashes will be necessary when small fish are examined. Look for the presence of motile or non-motile bacterial rods and fungal hyphae. The coverslip may be removed and the squash Gram stained for confirmation of bacteria as described for gill tissues.
- A squash of lesion material from a visceral organ or organs may be warranted if present and if its cause is not readily discernible. Gram stain and/or Geimsa stain of this material may also be warranted.
- If the cause of mortality or morbidity is in question as to whether or not the above procedures will provide an answer, histology samples should be taken as a backup measure, but only if moribund fish are available. Fish that have been dead for several hours or longer are generally not suitable for histology due to postmortem tissue autolysis. If fry are involved, whole fish may be dropped into Bouin's fixative or 10% buffered formalin. Fingerlings should have the abdomens opened with scissors for better fixative penetration.

- If clinical signs suggest a central nervous system disorder the top of the cranial cavity should be opened and the brain included in bacteriologic sampling using TSA. Heads from additional affected fish should be severed behind the gill opercula and placed into fixative for later histological sectioning of the brain.
- This necropsy procedure should include at least 5-10 moribund or otherwise affected fish. Control or healthy fish should be requested for comparison of whether abnormalities perceived are real or not. The number of control fish processed will depend upon the particular case and may range from 10 to none.

Histopathology procedures for finfishes: Due to the rapid rate of autolysis of fish tissues compared to that of homeotherms, they must be handled rapidly to prevent degenerative changes within the specimen, making ultimate diagnosis either unreliable or impossible. For satisfactory histological preparations, only freshly killed or moribund fish should be considered. Proper fixation is fundamental to all satisfactory histological preparations. The primary objective of fixation is to preserve the morphology of the tissues in a condition as near as possible to that existing during life. The most widely used fixatives are formaldehyde and Bouin's fixative. Small fish/fry can be directly dropped into the fixative while in the case of larger samples, incisions should made into the abdomen so that the fixative reaches the internal organs/viscera.

Histology of Bivalves: Bivalves less than 6 cm in length (shucked) can be fixed whole by dropping into preservative. Animals must be shucked cleanly from the shell by severing adductor muscles prior to fixation. For good fixation, larger bivalves require 3 incisions (anterior, mid, posterior) made across the surface of the animal about mid-way through the tissues. Do not cut completely through the animal so that individual specimens remain intact and tissues do not become mixed. Fixatives used for bivalves are Helly's fixative, Bouin's fixative or Davidson's fixative. After fixation bivalve tissues are firm enough for further processing. Each animal body is cut through the anterior, middle and posterior areas resulting in 4 separate pieces of tissue. One section, about 2 mm in thickness, is shaved with a razor blade from each of the faces of the tissues representing the 3 major body areas. The sections are placed within 1-3 tissue cassettes depending upon their size for dehydration and embedment. Small bivalves can either be embedded whole or cut longitudinally on the median axis and both tissue halves placed face down within a cassette.

Bivalve Larvae: Fix in a test tube of Helly's fixative, then centrifuge @ 1,500 rpm for 10 minutes. Discard supernatant and embed the larvae in an agar plug. Remove the plug from the test tube for dehydration and embedding in wax in the usual manner (trim if necessary).

Histology of Shrimp: The chitinous exoskeleton of shrimp prevents adequate penetration of any fixative by simple immersion. Consequently, the fixative must be injected into strategic internal areas of each animal prior to dropping the whole shrimp into the fixative. Inject fixative into the shrimp using a 10-ml syringe and appropriately sized needle, depending upon the size of the animal (small shrimp; i.e., small-gauge needle). Immediately after injection, slit the cuticle of the animal from the last (6th) abdominal segment to the base of the rostrum. The incision in the cephalothoracic region should be just lateral to the dorsal midline and that in the abdominal region should be mid-lateral. Do not cut too deeply into the underlying tissue. The objective is to break the cuticle to allow fixative penetration. After injection and body incisions, the animal may be dropped into the fixative. Transfer

shrimp to 70% ethyl alcohol after 48 hrs. Commonly used fixatives for shellfish: Davidson's fixative, Buffered formalin & Helly's Fixative.

Always ensure that the volume of the fixative is at least ten times that of the tissues. The fixed tissues are washed, dehydrated in ethanol series, cleared, embedded in paraffin and sectioned at 5-7 µm thickness. The sections are then stained with Haematoxylin & eosin and observed under the microscope.

Routine screeing of fish for parasites

Whenever possible examine fresh material. Fish should be freshly killed, without anaesthetic, and kept moist throughout examination, the reasons being

- Parasites are more easily recognised and identified.
- Parasites, especially ectoparasites, may leave the host or die after death.
- Collection of blood parasites is nearly impossible after death.
- Decomposition starts immediately after death and internal parasites may be destroyed by host's enzymes.

Other important points to be noted are:

- Handle fish as little as possible.
- Kill fish by cutting though cranium or though spinal cord immediately behind the head.
- Fish should be kept WET at all times during examination.
- If the fish is already dead, refrigerate, but keep moist. Do not freeze as most small parasite become unrecognisable and only large helminths and crustacea can be recovered. If examination is to be delayed, fix in 10% Formol saline and slit open the body cavity to allow fixative to penetrate internal organs.
- It is essential to examine skin and gills for ectoparasitic protozoa immediately after death as these may die or leave the host within a short time, e.g. flagellates.

Examination procedure

Kill fish quickly by cutting through the spinal cord with a sharp scalpel in the region immediately posterior to the gills.

Blood can be collected at this stage from the heart of major vessels using a Pasteur pipette. Place a few drops on a slide and allow to clot. A smear can also be made, fixed in methanol for 10 minutes and stained later.

1. Examination of skin

i. Take "scrapings" for high power examination (several, if fish is large). Scrape with a sharp scalpel in an 'anterior to posterior' direction and place mucus and epithelial cells on a slide in a drop of water. Avoid scraping scales as these reduce the visibility of small protozoa. *Thin preparations* are essential. Spread scrapings thinly and cover with a coverslip. Examine under high power.

NB. Scrapings should be made along the dorsum in an anterior to posterior direction including head, from the fins and from any discoloured areas or lesions.

ii. Examine the entire fish under low power using a stereo microcope. Be sure to examine under fins as well as other areas. Large metazoan parasites and *Argulus* can be seen in this way.

2. Examination of gills

i. Remove operculum and examine inside.

ii. Remove a whole gill and place on a slide or in a petri dish (add water if necessary and examine under low power on the stereo microscope. Separate the primary lamellae with needles to observe large monogenea and crustacea. Examine any lesions in detail.

iii. Cut off lamellae and remove gill arch. Place lamellae on a slide and spread thinly, chop if necessary and cover with coverslip. Examine under high power.

3. Other organs

i. Make incision along the ventral region. Remove abdominal wall to expose viscera. Examine visceral surfaces, abdominal cavity and pericardial cavity carefully under low power using stereo microscope. Examine any abnormalities or cysts, spots etc. in detail under high power.

ii. Remove alimentary canal and associated organs by cutting across oesophagus and around anus. Divide alimentary canal into stomach, pyloric caeca, fore,-mid and hind-intestine, and rectum. Examine surface and scrape contents onto a slide. Examine contents under high power. Compress sections of alimentary canal between slides and examine under High Power.

iii. Dissect and make squash preparations from heart, liver, gallbladder, spleen, kidney, gonads, urinary bladder and swim bladder.

iv. Dissect out eyes and open nares. Examine under low Power and high power for helminths. Squash lens and examine for eye flukes. Dissect out the separate tissues of the eyes carefully to determine the location as the site is helpful for identification.

v. Remove skin and slice muscle for helminth larvae and protozoan cysts. Squash muscle between slides or glass plates.

vi. Open cranial cavity, examine and make smear of brain tissue.

Fixation of parasites

The most commonly used fixative for preserving and storing parasites include alcohol-formoi-acetic (AFA or Davidson's Fixative). Bouin's, formalin and glycerine alcohol (Humason 1979). Formalin is probably the most commonly used and preferred fixative. Preservation in cold fixatives is not recommended because most parasites will contract and make identifications difficult or impossible.

Prior to fixation, worms should be thoroughly washed in saline and cleansed of mucus.

Protozoa: For Myxosporea: cut out the cyst with enough adjacent tissue and place in 10% formalin. For trophozoites of motile forms, place as many protozoa as possible on a clean microscope slide, add one drop of PVA-AFA (polyvinyl alcohol-acetic acid formalin alcohol) fixative adhesive, mix. spread over slide and allow to dry. Also, protozoans can be transferred to a vial of 10% formalin (keep in mind they will usually shrink).

Monogeneans/Trematodes: Trematodes should be transferred to a small glass petri dish. Remove excess saline or water. Heat 10% formalin or AFA to 85-90°C (begins to steam but not boil) in a fume hood. Add hot fixative to dish containing trematodes. For thicker worms, flatten under a coverslip and flood with warm fixative. For monogenea, drop infected gills into 10% formalin. Larger monogenea can be removed and fixed under light coverslip pressure.

Cestodes: Procedures are similar to that of trematodes; kill in 80'C water or formalin and store in buffered 10% formalin.

Nematodes: Kill in warm (80^0C) glycerine alcohol (1 part glycerine:3 parts 95% ethanol) and transfer to cold glycerine alcohol for storage.

Acanthocephala : For acanthocephalans it is necessary to evert the proboscis prior to fixation. Place worms in distilled water and refrigerate overnight. Transfer to warm 10% formalin or AFA.

Leeches: Fix in warm 10% formalin, or if very thick, flatten between two slides and flood with 10% formalin.

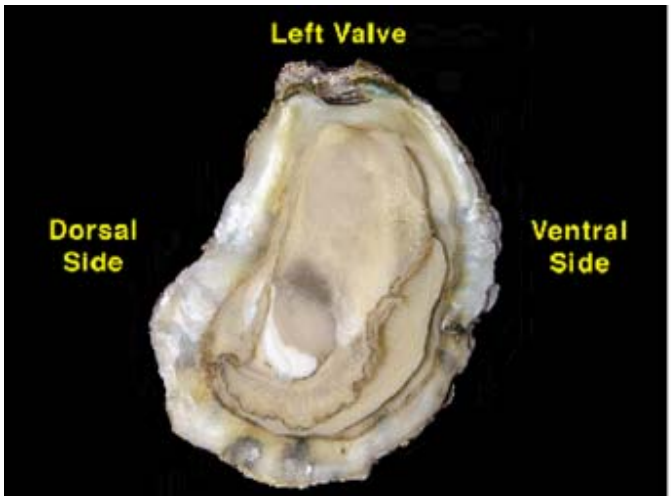
Copepods: Remove copepods. if possible, and drop into glycerin alcohol or 70% ethanol. If not easily detached, cut out a small piece of tissue containing parasite and place m 10% formalin or 70% ethanol.

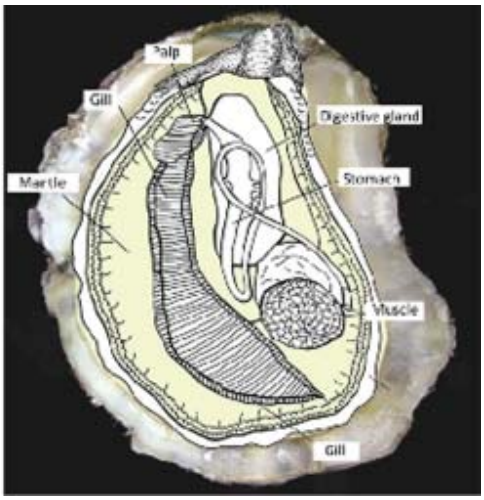
Routine screeing of bivalves

Oysters can be transported live from the collection site to the laboratory by keeping them wet by covering with a wet gunny cloth or cotton. In the lab, detach oyster individually, clean the sediment and debris from the shell by washing under tap water.

Orienting and opening the shells: Shell is composed of two unequal valves; the upper, flatter right valve and the lower, concave left valve which is cemented to the substratum and holds the animal. Orient the oyster pointing the anterior end (hinge region) away from you. Right valve or upper valve is opened by inserting a knife and cutting the adductor muscle. Detach the right valve leaving the soft tissue in the left valve.

Locating the organs: Tissue layer above is the right mantle skirt, the water space enclosed between the two mantles is the mantle cavity. Posterior adductor muscle can be observed in the





mantle skirt. Lifting the right mantle exposes the gills. The pericardial cavity and the heart lie anterior to the adductor muscle. On the ventral side (on our right) gills labial palp mouth etc can be located and on the dorsal side (on our left) rectum and anus. Visceral mass fills the space between hinge and the adductor muscle. In reproductive individuals gonads fill space in the visceral mass between stomach and digestive ceca.

The condition of the oysters are assessed by observing their general appearance, fouling, shell damage, presence of abnormalities, gaping, retraction of mantle, wateriness of the tissues, abnormal coloration, presence of abscess, lesions, pustules, tissue discoloration etc.

Morphometry and condition index (CI): general health and physiological state of the oyster can be assessed by the CI value. Morphometric features like dorso-ventral (length), anterior-posterior (height) and width (thickness) measurements are taken along with the total (shell on) weight, tissue weight and shell weight. Several formulas are in use for assessing the CI value like

CI= (dry soft tissue weight (g)*1000)/internal shell cavity volume (ml)

CI=Tissue weight (g)/(length(mm)*height(mm)*width(mm)*1000)

Tissue condition can be graded according to the table below

Condition index	Code No	Oyster appearance
Very good	1	Animal firm and filling shell cavity; creamy white and evenly textured; usually ready to spawn
Good	2	Not quite as firm or large as above; usually ready to spawn
Good minus	3	Coloration less opaque, often slightly yellow or grey
Fair plus	4	Animal distinctly not filling shell cavity; coloration often mottled, with blood vessels and muscle fibers showing through the more translucent epithelium
Fair	5	Oyster well developed but not opaque or tending towards white; grayish and translucent; flesh flaccid
Fair minus	6	Translucency more pronounced
Poor plus	7	Oyster not well developed, darker grey, often greenish ;pericardial cavity clear ;small portion of shell cavity filled
poor	8	Negative qualities more accentuated
Very poor	9	Animal distinctly atrophied; coloration dark and uneven, very translucent ;seldom more than third of shell cavity occupied; adductor muscle often discolored and transparent even in the normally white sector

Pathological observations

Shell observation: External shell is observed and the presence of foulers and borers are noted. *Polydora* is the major shell borer encountered and, barnacles, ascidians, mussels etc are

frequently observed foulers. Inside shell is viewed for any calcareous growths or discolorations. Heavy *Polydora* infections produce black discoloration, brittleness, foul smell and mud blisters are also visible in certain cases.

Tissue observation: the general appearance and condition of the tissue is noted. Usually a creamy white plumpy appearance observed in case of good condition and reproductive phase. But tissue appear dirty white and watery when it is in spent condition or diseased.

The mantle cavity of the animal is examined for the presence of parasites/ commensals. Mantle fluid is collected using a Pastuer pipette from the mantle cavity and a drop is placed on a clean alcohol wiped slide and observed under microscope after placing a cover slip. A number of protozoan ciliates and other algae etc can be found. Small pieces of mantle, gill and digestive gland tissues are squashed and examined for the presence of parasites.

Collecting hemolymph: After shucking the oyster excess mantle fluid is drained off. Using a sterile syringe (1ml, 27 gauge) 1-2 ml hemolymph is collected either from pericardial cavity or from the adductor muscle sinuses. Hemolymph collected is stored at 4^0c until observation. Hemolymph is mixed with May Grunwald's eosin-methylene blue solution and kept aside to stain the cells and later observed under microscope. There are three major types of hemocytes in oyster namely granulocyte, semigranulocyte and agranulocyte which can be identified after staining.

Ray's Fluid Thioglycollate Medium (RFTM) Culture for detecting Perkinsus infections:

Tissue samples from gill and mantle measuring approximately five to 10 mm are excised and placed in separate 30 ml tubes containing 15 ml fluid thioglycollate medium supplemented with 500 ìg ml^{-1} streptomycin and 500 U ml^{-1} penicillin-G potassium. The tubes were incubated at 24°C for 7 days, in the dark. After incubation, the fragments of tissue from each tube are collected, placed on a glass slide and macerated along with a drop of Lugol's iodine solution. The preparation was covered with a cover-slip, allowed to sit for 10 minutes and examined under the microscope.

Molecular diagnostic studies: Bivalve tissues for molecular diagnostic studies are fixed in 95% ethanol and stored under refrigeration. DNA extraction done using standard phenol/chloroform protocol followed by ethanol precipitation. This will be followed by PCR screening for major pathogens viz *Perkinsus.*

Suggested Reading

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Electron Microscopy in Disease Diagnosis

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Understanding the pathogen and the pathogenesis at cellular levels are imperative in the studies of disease causing organisms. With its very high resolving and magnifying powers, Electron Microscopy has opened up new vistas in studying the ultra structure and has become an indispensable tool in understanding many of the diseases and their etiological agents. The limitations of Light Microscopes, low magnifying and resolving powers (1000 x magnification and a resolution of 0.2 micrometers) paved the way for the development of electron microscopes. Electron Microscopes are instruments that use a beam of highly energetic electrons to examine objects on a very fine scale and function exactly like their optical counterparts. Present day electron microscopes are capable of giving magnifications up to 1000000 X and 800000 X and a resolving power of 0.1 nm and 0.4 nm in T E M and S E M respectively. Transmission electron microscopy (TEM) can reveal the ultra structural details at cellular levels, whereas Scanning electron microscopy (SEM) can show the morphology of minute structures/organisms in its three-dimensional state. Combining the TEM and SEM, it has become possible to study and classify the viruses and virus like organisms.

Commonly employed methods for disease diagnosis include histology, serology, microbiology, molecular diagnostics and electron microscopy and each method has its own advantages and disadvantages.

Pathogens	**Size**	**Microscopy**
Helminth	mm - cm	Light microscopy
Helminth eggs	50 mm and above	Light microscopy
Fungi	5 mm and above	L M & E M
Protozoa	2 mm and above	L M & E M
Bacteria	0.2 mm and above	L M & E M
Rickettsias	0.3 – 0.6 mm	L M & E M
Virus	0.01 – 0.4 mm (10 – 400 nm)	Electron microscopy

Among these diagnostic techniques, electron microscopy remains the most important tool to establish a viral etiology in the case of disease outbreaks without any previous history, and stands out as the only technique, which can visualize and record viral pathogenesis at cellular levels.

Histology uses light microscopy and is still an invaluable tool in disease diagnosis. It does not require sophisticated instruments and is useful in many disease conditions. However, misleading

observations may make confirmatory diagnosis difficult. In the case of viral infections, one can find lesions or inclusions, which are only suggestive of a specific viral infection through histopathology. TEM provides information about the morphology of pathogens, sub cellular changes / particles / structures etc. Moreover, due to the limited magnification and resolution, ultra structural / sub cellular changes and minute pathogens/stages cannot be observed.

Sero-diagnostic methods play an important role in disease diagnosis, especially in field conditions. Serology still remains the mainstay of viral diagnosis. The tests are normally based on specific antibodies (immunoprobes) and can detect sub clinical / latent / carrier states of infection. However, the draw backs of serological tests are (a) highly variable sensitivity & specificity (b) many viruses often produce clinical disease before the appearance of antibodies (c) Less useful in the case of latent viruses (d) antigenic cross-reactivity between related viruses may lead to false positive results and (e) less effective in invertebrates which does not produce antibodies.

Microbiological methods are widely used for the diagnosis of bacterial infections and involve culture, isolation and identification of the pathogens. But the procedure is tedious and time consuming and may even take weeks in some cases.

Molecular biology tools involve the detection of genetic material of pathogens using molecular probes. Advantages of Molecular tools include (a) extremely high sensitivity (b) easy to set up and (c) fast turnaround time. Disadvantages are (a) expensive (b) extremely liable to contamination (c) high degree of operator skill required (d) quantitative assay difficult and (e) difficulty in interpreting positive results, especially with latent viruses and (f) though they are more sensitive, are only capable of identifying the presence of genomic material for previously identified agents.

Electron microscopy can be an important adjunct to conventional culture and serologic techniques in diagnosing viral illnesses. Though detection of viruses by E M requires relatively large numbers of virions, and provides no information regarding specific serotypes within a virus family, it has the distinct advantages of being simple and rapid. Also, infectious particles are not required. Some viruses do not grow in tissue culture or grow only after special manipulation, and may not survive if transportation conditions to the lab are not optimal. Naturally, culturing would miss these agents. Additionally, a wide variety of agents can be visualized by E M; because specific reagents such as antibodies, antigens, or nucleic acid and protein probes are not required, one is not limited to the availability of these reagents, and prior knowledge of the virus identity for reagent selection is not required. Diagnostic electron microscopy has two advantages over enzyme-linked immunosorbent assay and nucleic acid amplification tests. After a simple and fast negative stain preparation, the undirected, “open view” of electron microscopy allows rapid morphologic identification and differential diagnosis of different agents contained in the specimen.

The biggest advantage of electron microscopy lies in the fact that it provides direct visual evidence of various pathogens/biological processes, while most of the other techniques are indirect and in some instances non-specific. Electron microscopic diagnosis is uniquely suited for rapid identification of infectious agents. A specimen can be ready for examination and an experienced virologist or technologist can identify, by electron microscopy, a viral pathogen morphologically within 10 minutes of arrival in the electron microscopy laboratory. Once the histopathological observations using light microscopy provides primary information on the target tissues, electron microscopy can

be employed to visualize the the pathogens and study its morphology. Electron microscopy can also provide information on the ultrastructural modifications/changes at sub-cellular levels caused by the pathogen.

So compared with other methods, E M benefits from an "open view", which means that as a "catch all" method it also reveals double infections and the presence of agents that might not otherwise have been considered. Finally, since the test entails the visualization of the virus itself, rather than a color change or agglutination reaction, false positive tests resulting from cross-reactions of reagents with similar materials are not likely. Hence electron microscope can be considered as the ultimate tool in identifying the etiology of emerging diseases.

Two types of preparations are primarily used for routine EM virus identification, negative staining and thin sectioning, although specialized research techniques such as scanning E M, specific antibody aggregation or labeling with electron-dense tags, *in situ* labeling, cryomicroscopy, and high-voltage microscopy have been used to classify viruses and describe virus-host relationships. With the simple negative staining preparation available, E M allows the rapid and direct detection of an etiological agent on a sample from a patient, or from diagnostic cell cultures.

Negative staining of liquid samples is very rapid, and can provide an answer within a few minutes to a couple of hours.. It enables the examiner to view cell particles and organelles in isolation. The isolated cell/particle is placed in a "puddle" of staining material, usually uranyl acetate or phosphotungstic acid, and is then supported on a thin, plastic film. The stain molecules deposit into surface crevices in the specimen during the drying process and typically produce a "ghost" image in which the specimen appears light against a dark background. Sensitivity and specificity of E M may be further enhanced by immuno electron microscopy, which includes classical immunoelectron microscopy and solid phase immuno electron microscopy.

In classical immuno electron microscopy, the sample is treated with specific anti-sera before being put up for EM. The viral particles present will be agglutinated and thus congregate together by the antibody, making them easily visible.

In solid phase immuno electron microscopy the grid is coated with specific anti-sera. The virus particles present in the sample will be absorbed onto the grid by the antibody thus enhancing the visibility under the microscope.

Advantages: The most important among the benefits offered by the electron microscope is undoubtedly the very high resolution. Since timely and accurate diagnosis forms the first step in the health management of farmed fishes and shellfishes, the right diagnosis defines the very success of disease control. Though E M has an important role in the diagnosis of viral infections, it is equally useful in the diagnosis and understanding the pathogens as well as the pathological changes caused by various other pathogenic organisms. As a confirmatory diagnostic method for many of the existing and emerging diseases, especially of viral origin, electron microscopy still remains an indispensable tool in the field of disease investigation and control. To exploit the potential of diagnostic electron microscopy fully, it should be quality controlled, applied as a frontline method, and be coordinated and run in parallel with other diagnostic techniques.

Disadvantages: However, the disadvantages of E M in the diagnosis of infections are (a) detection of viruses by E M requires relatively large numbers of virus particles (b) possibility of false negatives, if concentration is very low (c) provides no information regarding specific serotypes within a virus family and (d). Factors like high cost of operation and infrastructure, need for skilled technical personnel, laborious and time-consuming procedures, thorough knowledge needed for interpretation etc. restricts the use of electron microscopy as a routine diagnostic tool.

Suggested Reading

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