Winter School on

'RECENT ADVANCES IN DIAGNOSIS AND MANAGEMENT OF DISEASES IN MARICULTURE'

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Course Manual

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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. 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 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 biology and electron microscopy. Each method has its own advantages and disadvantages and the choice depends on various factors, including the nature of the disease. Among the spectrum of 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, at least in some cases, misleading observations may make confirmatory diagnosis difficult. More over, due to the limited magnification and resolution ultra structural / sub cellular changes cannot be observed.

Serodiagnostic 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. A battery of serological tests is available and most of them are cheap when compared to others. How ever, 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 and (d) antigenic cross-reactivity between related viruses may lead to false positive results.

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

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.

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

Electron microscopy provides direct visual evidence of various pathogens / biological processes, while most of the other techniques are indirect and in some instances non-specific. 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.

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. 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 those that do 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. So compared to other methods, E M benefits from an "open view", which means that as a catchall method it also reveals double infections and the presence of agents that might not otherwise have 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.

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, organelles, and molecules in isolation. The isolated cell particle or molecule 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.

Thus TEM is a very useful tool for providing rapid ante-mortem and post-mortem diagnoses of various viral infections through the examination of appropriately processed lesions, biopsies, and excreta. How ever. the disadvantages of E M in the diagnosis of viral 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 and (c) provides no information regarding specific serotypes within a virus family.

Negative staining

A thin support film of carbon coated Formvar or collodion is placed over the EM grid. Liquid samples are first cleared of large debris by centrifugation, a drop of the supernatant is placed on a parafilm and a grid with a carbon-coated support film is placed on the drop under a small inverted Petri dish for 5-10 minutes. The grid is then drained and negatively stained by placing it, specimen side down, on a drop of saturated aqueous uranyl acetate or 2% aqueous phosphotungstic acid for 30-60 seconds. The preparation is drained and viewed immediately in TEM. If no viruses are found, the sample is concentrated (ultra- centrifugation), stained and observed using the same procedure.

Solid tissue sample preparation requires more time, but results can usually be obtained within a few days.

Sampling

Immediately after the death of the organism, post mortem changes will takes place making the tissue unsuitable for ultra structure studies. Hence for electron microscopy, live animals are always preferred. The animals are sacrificed, the desired tissues/samples dissected out and immediately placed in cold fixative. The desired size of the tissue to achieve proper fixation is about 1 mm. Small animals and larvae of less than 2 mm size are fixed in ice-cold fixative as a whole in live condition. The sample vial should be labeled properly.

Fixatives

Fixatives help to preserve the structures in the living cell and prevent the changes induced by autolysis. There is no single ideal fixative and so a combination of fixatives is preferred depending on the type and nature of the tissues. In electron microscopy, 2 – 4 % Gluteraldehyde is used as the primary fixative which is excellent in fixing nucleic acids, nuclear proteins and carbohydrates but not lipids. Poor contrast and slow penetration are the limiting factors of gluteraldehyde fixative. Osmium tetroxide is used for secondary fixation. It acts as both fixative as well as stain, fixes nucleic acids, carbohydrates and lipids and provides contrast and fast penetration. The combination of gluteraldehyde and Osmium tetroxide as primary and secondary fixatives, gives the desired results in contrast and resolution.

Fixatives are prepared in a suitable buffer for two reasons, to maintain the pH (7.2 to 7.4) and to maintain the osmolality, in order to minimize the swelling or shrinkage of the tissues which may otherwise lead to artifacts. The most commonly used buffer is Sodium Cacodylate buffer.

Primary fixation

Tissues are fixed in 2 to 4% Gluteraldehyde in 0.1 M Cacodylate buffer (in the case of marine species, 5 to 3% NaCl or sucrose can be added to the fixative). For proper penetration of the fixative, the tissues should not exceed 1 mm in size. Fixation is carried out for 4 - 6 hrs (varies depending on the nature of the tissues), at 4 °C. After fixation, the fixative is drained and tissues washed thrice (15 mts each) with buffer. In case of larger tissues, further trimming is done if required and washed with fresh buffer.

Secondary Fixation or Post fixation

For secondary or post fixation, the washed tissues are transferred to 1% Osmium tetroxide (OsO_4) in 0.1 M cacodylate buffer, kept for 1- 2 hrs at 4 °C (above 4 °C, OsO_4 disintegrates). OsO_4 treatment turns the tissues black. OsO_4 is drained and tissues washed two to three times with buffer, for 15 min each, or until free of a black precipitate formed from excess OsO_4. Samples can be stored in buffer under refrigeration until further processing is desired. (Since OsO_4 is highly toxic, care must be taken while handling. Always use gloves and carry out all operations under a hood).

Dehydration

Dehydration is done through graded alcohol or acetone series to remove the water from the tissues.

Dehydration can be done as follows:

30% Acetone (analar)	two changes, 15 mts each at 4°C
50% Acetone (analar)	two changes, 15 mts each at 4°C
70% Acetone (analar)	two changes, 15min each at 4°C, (tissues can be stored in 70% acetone indefinitely, until further processing)
80% Acetone (analar)	two changes, 15 mts each at 4°C
90% Acetone (analar)	two changes, 15 mts each at 4°C
95% Acetone (analar)	two changes, 15min each, 4°C
100% Acetone (analar)	two changes, 15 min each, 4°C
100% Acetone (analar)	two changes, 30 mts each at room temperature
Propylene oxide	two changes of 15 min each at room temperature

Infiltration and Embedding

Fixed and dehydrated tissues are infiltrated with liquid plastic resins and then cast into blocks. The purpose of embedding is to allow future ultra thin sectioning of the material. Commercially available Plastic resins like Epon or Spurr are used for embedding. The media is mixed as per the instructions under a fume hood. Prepare fresh media 2-3 hrs. prior to use, as it will absorb the water vapor from the surroundings and the components will begin to polymerize. Mixture of embedding medium (Spurr's medium) and acetone is prepared in various grades (mix. A – medium and acetone in the ratio 1: 3, mix. B – medium and acetone in the ratio 2 : 2 and mix. C – medium and acetone in the ratio 3 : 1) and the tissue kept in each for 1 – 2 hrs each or as specified (period varies with the medium used) for infiltration. For embedding, medium is prepared as instructed, poured into readymade moulds made of plastic or silicon rubber and infiltrated tissues transferred to it, taking care not to trap any air bubbles. The moulds are then kept in an incubator at 70 °C for 12 to 24 hrs.

Each tissue with reference to the experimental objective requires an evaluation of the methods, subjected to a careful examination of pertinent literature. There is no schedule that will work for all tissues and conditions.

Trimming

The resin blocks are trimmed to remove the unwanted areas using a glass knife fitted to an Ultramicrotome.

Sectioning and staining

To achieve high resolution for electron microscopy, the sections should be very thin (60 nm) and are prepared using an ultramicrotome. Standard procedures are followed for obtaining semi thin and ultra thin sections for light and electron microscopy respectively. Semi thin sections are first taken, stained and observed under a light microscope for determining the area for ultra thin sectioning. The blocks are again trimmed and ultra thin sections taken. These sections are floated on distilled water, stretched to remove the wrinkles and collected over the matty/dull surface of the copper or nickel grid.

Staining

Double staining with Uranyl acetate and Lead citrate is employed for routine electron microscopy studies. The sections are first stained with Uranyl acetate. A drop of Uranyl acetate (saturated solution in 50% ethanol) is taken on a clean glass slide and the grid with the section side down is kept on to the stain drop and is covered with any opaque object to ensure darkness to carry out the staining effectively. After 10 – 15 mts, the grid is taken out and washed 3 - 4 times in double distilled water (ensuring that the sections are not washed away) and dried with a filter paper. The grids are then stained with Lead citrate for 1 – 4 mts., washed well and dried. In the case of particulate specimen, the specimen is taken on formvar-coated grids, subjected to negative staining using 1-3% Phosphotungstic acid and dried.

Observation and photography

The grid carrying the stained section is loaded into the electron microscope, the image observed and recorded on photographic plates/film. In order to study and interpret EM results one has to have a thorough knowledge about the ultrastructure of the normal cells and the pathogen.

Specimen preparation for S E M

The specimen is first fixed with gluteraldehyde as in the case of TEM and washed well in buffer. Post fixation with OsO_4 is optional. The specimen is subjected to dehydration using ascending grades of acetone as in TEM processing. The dehydrated specimen for SEM has to be dried without causing any shrinkage. Except in the case of fine particulate specimen, critical point drying or freeze-drying is usually preferred for drying SEM samples. The dried specimen is then coated with a thin conductive metal film (Gold, Palladium etc.) using an ion coater to prevent charging artifacts and to stabilize the specimen mechanically.

Presently, variable pressure SEM s are available which can operate without high vacuum thus avoiding the time consuming specimen preparation techniques as well as reduce specimen damage caused during coating. SEM provides valuable information on the surface morphology of various structures, pathogens / spores etc.

Benefits of electron microscopy

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. 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, but 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.

References

1. Bozzola, J. J., and L. D. Russell. 1992. Electron Microscopy. Jones and Bartlett, Boston, MA. 542 pp.
2. Dawes, C. J. 1971. Biological Techniques in Electron Microscopy. Barnes and Noble Inc., New York. 193 pp.
3. Doane, F. W., and N. Anderson. 1987. Electron Microscopy and Diagnostic Virology. Cambridge University Press, Cambridge. 178 pp.
4. Hayat, M. A. 1989. Principles and Techniques of Electron Microscopy: Biological Applications. CRC Press, Boca Raton, FL 469 pp.
5. Hayat, M. A., and S. E. Miller. 1990. Negative Staining. McGraw Hill, New York. 255 pp.
6. Hsiung, G. D. 1982. Diagnostic Virology. Yale University Press. New Haven and London. 276 pp.