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Diagnostic Electron Microscopy (EM) for Avian Diseases - An Overview

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Abstract: ***Electron Microscopy (TEM and SEM) is one of the gold standard techniques to study the patho-morphalogical changes of different diseases in the field of life science. EM is otherwise called as "cell pathology" which deals with all membranous structural deviations, localization of disease causing agents, attachment of virions / viruses to the cilia, villi, and other membrane structures of the different cells. There are various techniques which involves for accurate diagnosis and specific pathogen identification but, EM technique is having its significance to support all molecular techniques due to meticulous observation of all sub-celluar structural changes in response to the disease causing agents which target different structures in a cell or group of cells. EM is a multi-stepped, time consuming and expertise technique which needs to have continuous practice and up date.***

Keywords: Electron microscopy, TEM, SEM, LM, ultrastructure, resins, Virus, specimen, glutaraldehyde, uranyl acetate and lead citrate

1. Introduction

In general, avian diseases are wide range beginning from nutritional, bacterial, viral, neoplastic, parasitic, mycotoxic and many other conditions are being recorded all over the globe. Most of the diseases are being diagnosed on the basis of pathgnomonic gross lesions conventional techniques like histopathology, IHC advanced tools like QRT-PCR techniques etc. The demonstration of minute pathogens like virus, and sub cellular structural alterations due to disease process are possible with Electron Microscopy (EM) only which is rapid, specific, and accurate with high resolution [(6,7)]. At this juncture it is necessary to value every tool used in the field of diseases diagnosis. Different avian viral pathogens like avian influenza, avian infectious bronchitis, avian pox, Newcastle disease, infectious bursal disease, chicken anemia etc., were demonstrated under EM by different authors [(2,4,6,7,12,13,18)]. Besides this pathogenic bacteria, mycoplasma, aflatoxin pathogens and probiotics attachment with host cells (epithelial cells of respiratory and gastrointestinal tracts) and its morphological studies are being demonstrated in experimental specimens and also in samples of natural out breaks by using EM at Ruska Labs. Viruses can be demonstrated under EM in different specimens like host tissue, experimental cell lines, tissue homogenates, experimental embryonic fluids, stool, tracheal swabs; intestinal fragments. Complexity of multiple pathogen interaction with host cell or experimental cell lines is possible only through EM. Hence, it is more relevant to brief about EM and different methods adopted for EM specimen preparation like ***resin embedding method and direct electron microscopy*** (rapid preparation/negative staining technique)[(13)] may be useful to all Avian Health Practitioners (AHP).

2. Introduction to Electron Microscopy (EM)

Electron Microscopy (EM) is a dynamic specialized tool to study the sub cellular structures and surface morphology of biological and non biological specimens by using a beam of electrons [(1, 10)]. In Light Microscopy (LM) visible light and optical (glass) lenses are used as a source of illumination to magnify the specimens (10 to 1,000). EM operates in vacuum and electron beam (e.g., $\lambda = 0.005$ nm) will act as a source of light to magnify the processed specimen through electromagnetic lenses when compared to the wavelengths of visible light (e.g., $\lambda = 400$ nm to 700 nm) [(5)]. In EM, resolving power is inversely proportional to the wavelength. In other words, increasing the velocity of electrons results in a shorter wavelength and increased resolution [(5)]. Research in development of electron microscopes began in the year 1920s. Under the guidance of ***Max Knoll, Ernst Ruska*** began work on the development of electron lenses (Germany, 1928). The first functional TEM was developed in the early 1930s by Ruska for which he was ***honored with Nobel Prize in Physics in 1986*** [(1, 5,9,11)]. Early EM studies primarily focused on optical behavior of electron beams under various conditions. Thus, no biological applications were initially envisioned. However, due to superior magnifying power of an EM it soon became clear that they could be applied to the study various biological specimens [(9,16)]. Presently EM remains an important and significant tool in diagnostic ultrastructural pathology besides physical and material science [(9,15)].

There are two basic types of electron microscopes (TEM and SEM) were invented within the same decade (SEM was invented by ***Manfred von Ardenne in 1938)***, but they differ fundamentally in their usage [(9)]. In brief, the TEM projects electrons through an ***ultrathin section*** of the specimen and produces a ***two dimensional image*** (up to 1000kx) while SEM generates ***three dimensional image*** (up to 100kx) image with the help of ***secondary electrons.*** Extreme high magnifications above 200,000 are rarely used by biologists [(3)]. EM allows investigators to detect the specimens in much greater detail than those examined under LM. **Conventional electron microscopy** is used today in many research laboratories, which are attached with computers to make a digital photography with CCD (charge coupled devise)

which makes analysis easy [9,10]. There is ample evidence in the published literature that EM has significantly contributed to our understanding of the ultrastructure of a variety of specimens, including those of pathogenic and nonpathogenic agents [16].

3. Resin Embedding Method Protocols (Multi-Stepped Protocol)

Specimen preparation for TEM includes eight major steps (multi-stepped): *slicing & cleaning, primary fixation, washing, secondary fixation, dehydration, infiltration with a transitional solvent & resin and embedding, polymerization, sectioning and staining.*

3.1 Slicing and cleaning the surface of the specimen

Proper cleaning of the surface to remove variety of unwanted deposits if any (not removed may get permanently fixed) otherwise impossible to remove later [1]. Bozzola and Russell suggested that the specimen should be quickly washed or rinsed in a suitable buffered solution (physiological pH) by the way of gentile swiping or washing for three times for 10 minutes at room temperature. The size of the specimen should be in mm^3.

3.2 Primary fixation or stabilization of the specimen

There are various stabilizers / fixatives (Aldehydes, osmium tetraoxide, tannic acid, or thiocarbohydrazide) are available to achieve this step [1,16]. For all biological samples including poultry simple chemical fixation is advocated (0.1M PBS/cacodylic buffer based 2.5% glutaraldehyde solution 10 times to the volume of tissue) and can be stored at room temperature or 4^0 C overnight / few hours / few days / few weeks / few months [1,16].

3.3 Washing /Rinsing of the specimen

In order to remove excess fixative from the samples, the specimen should be thoroughly washed twice or thrice with 0.1 M PBS/cacodylic acid buffer (pH 7.3) for 20 minutes [1].

3.4 Secondary fixation / postfixation / staining of the specimen

Secondary fixation is crucial to stain the specimen (block color) and to protect the specimen during other steps employed such as embedding, sectioning. This step also helps in avoiding artifacts and helps in conductivity of electrons to generate quality image [1]. All biological samples can be successfully stabilized for TEM investigation by post fixation with 1% aqueous osmium tetraoxide or 0.1M cacodylic acid buffer (pH 7.3) for 1 to 2 hrs at room temperature (rehydration). It is important to realize that no fixation procedure is ideal; any type of fixation is likely to cause some alterations in the specimen [1].

3.5 Dehydration of the specimen

The rehydrated sample should be dehydrated in a graded series of ethanol. More specifically, the following protocol is useful: A series of 50, 70, 80, 90, and 99.9 percent ethanol, each for 45 minutes at room temperature. If time does not permitting these can be stopped at 70% level and stored at 4^0C. This process allows the water in the samples to be slowly exchanged through liquids with lower surface tensions [1, 10].

3.6 Infiltration of the specimen with a transitional solvent

The ethanol is not miscible with the plastic embedding medium hence it should be replaced with another intermediary solvent like propylene oxide is necessary[1]. Immersion in propylene oxide twice for 20 minutes at room temperature. Alternatively 1:1, 1:2 and 1:3 ratio of ethanol and resin can be used each for 30 minutes finally replaced with pure resin for overnight and keep it in 4^0C. This process facilitates better sectioning and EM details will be more clear [1,10].

3.7 Infiltration with resin and embedding of the specimen

All biological specimens can be embedded in a variety of different media depending on the purpose (conventional TEM or immuno TEM). For conventional TEM, the epoxy resin EMbed, araldyte or LR white or Durcupan ACM are quite suitable. Next day of post transitional step, the specimens should be immersed in a freshly prepared pure resin and left for 2 hrs at room temperature followed by embedding (free of air bubbles), block making and keep it for polymerization at 50^0C to 60^0C for 48 to 72 hrs. Store the samples in desiccater for 3-5 days or place it for 1-2 weeks at room temperature which will improve the subsequent trimming and sectioning quality as the resin blocks continue to harden during this time[1].

3.8 Sectioning and staining of the specimen

The procedure of cutting of the specimens into semi thin and ultrathin slices (sections) is known as microtomy and ultramicrotomy, respectively [1]. Semi thin sections (about 600 to 800 nm) should typically be stained with toluidine blue for 1 min on a hot plate (70^0 C to 90^0 C), examined under LM, and used for identifying the specimen within the resin block before proceeding with ultramicrotomy. Ultrathin sections (about 50 nm to 60 nm) should typically be stained with saturated uranyl acetate (20 minutes) followed by lead citrate (for 5 minutes)[1].

As mentioned before, TEM sample preparation is multi-stepped; every step can virtually affect the quality of the final electron micrograph. It is therefore important that the expert should plan and execute meticulously. ***I believe that these procedures involve a significant time commitment and require knowledge, patience and skills that come only through practice***. It is important to note that most of the chemicals used in EM are dangerous. All steps should be under fume hood on orbital shaker. Bozzola and Russell wrote an laudable chapter on safety in the EM laboratory. They emphasized on the importance of training in the proper usage of all equipments and reagents in the EM laboratory. They also mentioned that the investigator must be aware of potential hazards such as fire, chemical, electrical, and

physical harm associated with these items. EM facilities usually offer training and orientation programs all over the world. In my opinion it is exceedingly recommend not only for the apprentice in EM (first learning) but also for the experienced investigator (continuous learning).
(Fig 1 to 3 are TEM images of cartilage cells in thiuram induced tibial dyschondroplasia in broilers).

4. Scanning Electron Microscopy

Scanning Electron Microscopy (SEM) is also a powerful tool for investigation of surface structures and multiple pathogen interactive studies of samples. This technique is advantageous to observe a large area of field at different focal points [(5)]. SEM also facilitates relatively wide range of magnification allowing the investigator to easily focus in/on an area of interest on a specimen that was initially scanned at a lower magnification. Furthermore, the 3-D images may be more appealing to the human eye than the 2-D images (obtained with TEM). Therefore, an investigator may find it easier to interpret SEM images. Finally, the number of steps involved for preparing specimens for SEM investigation is lower and less time consuming than the TEM sample preparation. However, SEM specimen preparation harbors various risk factors that can easily distort the integrity and ultrastructure of the sample. The basic steps involved in SEM sample preparation include *thin slicing, surface cleaning, stabilizing the sample with a fixative, washing /rinsing, dehydrating, drying, mounting of the specimen on a metal holder over double sided carbon conductivity tape, and coating the sample with a layer of a material(e.g., gold, gold-palladium or platinum) that is electrically conductive*[(1)]. Because each of these steps are crucial and will affect the outcome of the study, they are described in more detail below. The first four steps are essentially the same as those described for TEM specimen preparation. These steps are therefore only briefly mentioned below.

4.1 Slicing and cleaning of the surface of the specimen

As discussed earlier, the best way to clean the surface of biological samples from contaminants is to carefully washed or rinsed. The sample size should in cm^3.

4.2 Fixation / Stabilizing of the specimen

All biological samples can be chemically prefixed by immersing the specimens in a 2.5% glutaraldehyde solution prepared in 0.1 M PBS /cacodylic acid buffer (pH 7.3) and stored as that of TEM samples.

4.3 Washing / rinsing of the specimen

In order to remove excess glutaraldehyde from the samples, should be subjected to a thorough washing or rinsing procedure in 0.1 M PBS /cacodylic acid buffer (pH 7.3) as explained for TEM sample preparation.

4.4 Dehydrating of the specimen

The dehydration process of a biological sample needs to be done very carefully. It is typically performed with either a graded series of acetone or ethanol. The protocol that proved most suitable for dehydrating the biological specimens for SEM includes the immersion of the specimens in 50% , 70% , 80% , 90% and 100% acetone (dried with $CaCl_2$) of each for 45 minute at room temperature under fume hood on orbital shaker. This process allows the water in the samples to be slowly exchanged through liquids with lower surface tensions [(1,5,10)].

4.5 Drying of the specimen

The scanning electron microscope also operates in a vacuum, for which specimens must be dry otherwise the sample will be destroyed in SEM chamber. Many electron microscopists consider a procedure called the Critical Point Drying (CPD) as the gold standard for SEM specimen drying by using liquid carbon dioxide in which specimen is dried without structural damage [(1,10)]. It is very important to follow exact instructions of the manufacturer of the CPD apparatus, to avoid significant structural alterations. In my experience, I tried a specimen drying process called Simple Desiccation (SD) and also Vacuum Desiccation (VD), which are giving superior results as excellent as that of CPD. This technique is essentially a simple air-drying procedure after fixation, rinsing, and dehydration of the specimens. SD/VD is risky as the specimen may collapse, flatten, or shrink or be rolled and become uncontrollable under these conditions[(1,10)]. Although SD / VD is faster and cheaper, this method is like ***"walking on a tight rope."*** For an EM beginner, I would suggest that the safer method is CPD. I would like to recommend the same procedure (But I have performed it only few times by using liquid carbon dioxide as the transitional fluid).

4.6 Mounting of the specimen

After complete drying of the samples, they must be mounted on metallic (Aluminum) stubs using a double sided sticky carbon conductivity tape. It is important that the investigator first decides about the best orientation of the specimen to be mounted before placing on carbon conductivity tape. A re-orientation proves difficult and can result significant damage to the sample. If it is not properly stuck to the tape a drop of pure silver paste can be used for its adherence.

4.7 Sputtering (coating) of the specimen

The idea of coating the specimen is to increase its conductivity in the SEM and to prevent the build-up of high voltage charges on the specimen by conducting the charge to the ground[(1)]. Typically, specimens are coated with a thin layer of (approximately 20 nm to 30 nm) a conductive metal (e.g., gold, gold-palladium, or platinum) for 180 seconds. In the Ruska Lab's, (College of Veterinary Science, PVNRTVU, Hyderabad, T.S), using gold, and found it most suitable. To guarantee best results (i.e., to achieve an even layer of metal coating over the sample), one should carefully follow the instructions of the sputter coater manufacturer. It is important to remember that each step has to be performed to perfection in order to achieve quality images that can be interpreted without the influence of artifacts caused by specimen handling. (Fig 4 to 10 are some the specimens in different experimental broilers and emu birds).

5. Suspensions for EM[8,14]

1) Centrifuge the suspension at a speed that will yield a solid pellet of the material under study.
2) Add the fixative slowly down the wall of the tube taking care not to dislodge the pellet.
3) Allow to fix for 10 min at room temperature and then release the pellet using a wooden cocktail stick and leave for a further 20 min. The material can now be treated as tissue blocks
4) If the pellet resuspends, the pellet can be recentrifuged after each part of the process. Or:
5) Resuspend in 1% low-gelling temperature agarose (37°C) in buffer, centrifuge to pellet, cool and cut into blocks and then proceed like embedding method.

6. Direct Electron Microscopy - Negative/ Positive Staining

Different materials like intestinal contents, stool, embryonic fluids etc., were demonstrated under direct EM by various methods described by different authors [13,18,14,8] by using negative sating and positive stains.

1) A drop of about 10 µl of the virus suspension to be studied is applied to the hydrophobic surface of a parafilm square in a Petri dish.
2) A formvar-coated grid is floated onto this drop for one minute, with the formvar side of the grid in contact with the liquid.
3) The excess liquid is removed from the grid by touching its border with a cut piece of filter paper.
4) The grid is immediately floated in a drop of 1.5% phosphotungstic acid, 2% ammonium molybdate or 1% aqueous uranyl acetate, depending on the specimen.
5) For a better assessment of the samples, two or three grids should be prepared, each stained with one of these stains.
6) After staining for one minute, the excess stain is removed with filter paper and the grid left to dry for a few minutes, before insertion into the microscope column.

7. General precautions in EM for interactive studies

Many factors will influence the EM studies of multiple interactive specimens:

1) Laboratory procedures need to be carefully reviewed as some drugs can affect the function and structure of host cells.
2) The procedures for the establishment of cell cultures and explants cultures can influence the EM data (e.g., improper collection of in-vivo/biopsy material by using surgical procedures)
3) Most host cells have surface exposed structures (e.g., cilia and microvilli) that can be easily damaged during handling; this damage may lead to misinterpretation of EM data.
4) Infection of host cells with specific pathogens may results in accumulation of exudates and cell debris which requires a balanced washing protocol.

Pathogen and host cell interactive studies may provide a voluminous knowledge about pathogenesis of any disease or apoptotic/necrotic changes due to ageing of the cell. The noteworthy feature in multiple pathogen interactive studies is that there is a direct relation between membranous structures of the host cells and pathogens which is very sensitive. Hence, proper care should be taken during processing of samples for EM studies.

8. Conclusions and future outlook

A descriptive approach of interactions between pathogens and host cells besides an exhaustive ultrastructural examination of different types of biological samples would not be possible without the electron microscope (EM). Both TEM and SEM have proven over the years to be valuable paraphernalia in this regard. Although TEM generates a different set of electron images than SEM, and thus provides different scientific data, the ***combination of these two methods in a single investigation can be extremely powerful besides other advanced techniques.*** Each technique will create its own milestones in the field of science hence; no technique should undermine the other. As per my experience, EM is not any easy technique unless one should train and gained handful experience to become expert which is possible with continuous practice only. But any biologist essentially a pathologist should have complete idea about EM techniques to narrate healthy and diseased cells. EM techniques are gaining pivotal importance in cancer biology, tissue culture biology, toxicological studies and many other emerging and challenging fields. There are many possibilities that an investigator may not get usable electron micrographs for interpretation. The reason behind this is multi-step specimen preparation for EM; a single mistake in one of these steps will affect all remaining steps, and outcome of entire work will go futile exercise. Despite the risk factors involved, TEM and SEM techniques provide fascinating images of biological specimens, in particular of the smallest free-living and self-replicating life forms on planet Earth. Finally, I wish that diversified specialists should enter the field of electron microscopy as this would allow exchanging of exigent ideas and thoughts. There are many new recent advances in electron imaging technology providing numerous new tools for viewing and characterizing pathogens (e.g., TEM with 3D tomography, Cryo EM and STEM). This should be enough reason to attract young scientists who develop an interest in ***"playing"*** with these powerful machines and applying the generated data to the fields of life science and pathology.

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Transmission electron micrographs: (Fig.1; Fig.2 and Fig.3)

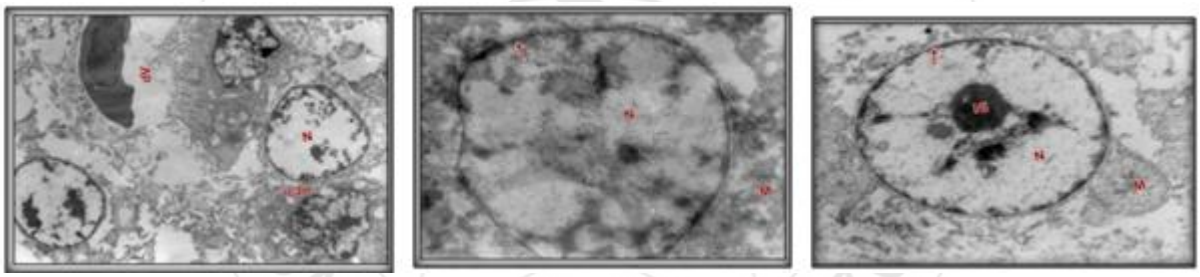
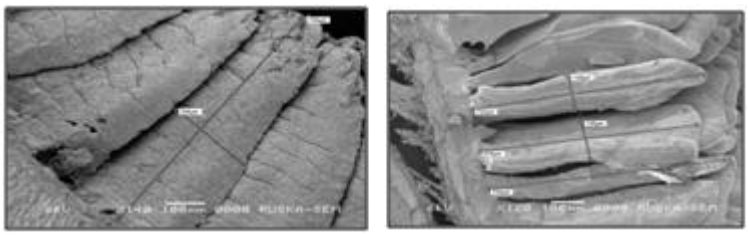


Figure 1: Normal cartilage cells showing dilated micro-capillaries (dv) and intercellular junctions(icjn) and nucleus (N). Urenyl acetate and Lad citrate (UA&LC). 66150x

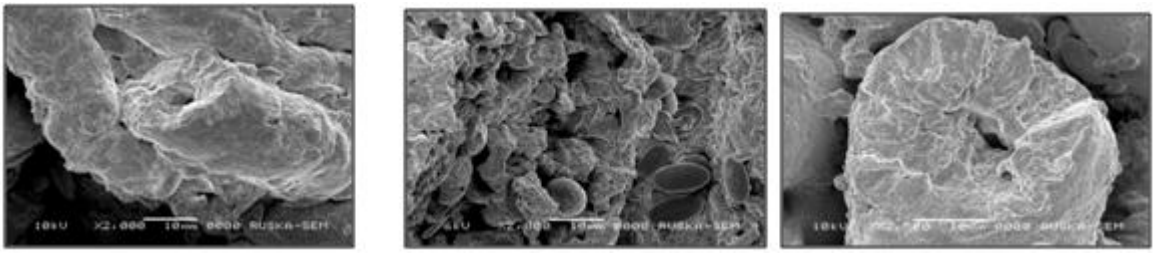
Fig.2: Abnormal cartilage cells(tibial dyschondroplasia) swollen nucleus (N) with margination of chromatin (arrow), swollen mitochondria (M) with mild dilation of nuclear membrane. UA&LC.15120x.

Fig. 3: Abnormal catrilage cell showing moderate swelling of nucleus (N) with mild margination of chromatin (arrow) centrally palced condensed Nucleolus (NL)and swollen mitochondria. UA&LC.15120x.

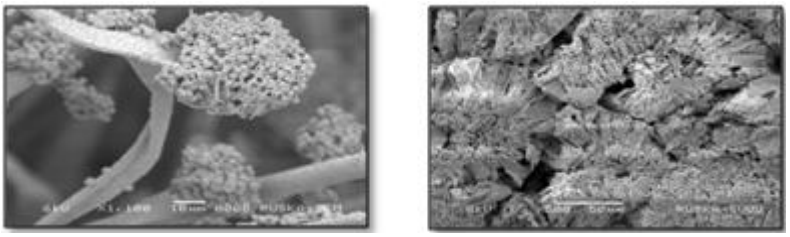
Scanning electron micrographs (Fig. 4; Fig.5; Fig. 6; Fig 7; Fig.8 Fig.9 and Fig 10)



Normal and distorted villi of Intestines



Normal renal tubules and hemorrhages and mild thickening of tubule



Aspergillum and Emu egg shell surface.