TEM of Paraffin-Embedded H&E-Stained Sections for Viral Diagnosis (An Unusual Papovavirus Case)

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Viral infections can be focal and therefore difficult to find by electron microscopy. In addition to sampling limitations, sometimes the only specimen available for examination is tissue that has already been prepared for light microscopy (LM). We have diagnosed a papovavirus infection in skin by embedding hematoxylin and eosin (H&E)-stained sections for ultrathin sectioning and transmission electron microscopy (TEM).

Immunosuppressed patients can have unusual infections or infections in unusual locations. These agents may be difficult to identify due to their not being suspected and incorrect tests being ordered. Molecular testing requires an idea of potential infectious agents for the selection of the proper probe (e.g., antibody, nucleic acid primers), and culture techniques require that the agent be able to grow *in vitro*. Electron microscopy provides a method of examination and identification that does not necessitate an *a priori* notion of the pathogen. Whatever is present can be visualized without requiring a specific reagent, assuming the observer can recognize the pathogen. At the very least, someone familiar with normal tissue will be able to recognize areas that appear different and can consult an expert or atlas for comparison.

Several methods have been published for enhancing the possibility of locating focal lesions and for processing thin specimens. Localization of focal infections in wet tissue can be achieved by confocal microscopy; excised areas are then processed for TEM [1]. Monolayers of cell cultures have been processed *in situ* for thin sections [2]. Wax can be removed from paraffin-embedded blocks for further processing of tissue for TEM and other studies [3, 4], but a focal infection might be missed. The processing of unstained frozen or wax sections (hence, unidentified lesions) for TEM has been described, but not for viral diagnosis [5]; this procedure is useful when lesions have been seen in stained LM sections and additional tissue is available for EM processing. The technique discussed here shows that some viruses can still be identified by TEM in tissue that has been processed through the harsh treatment for LM processing and examination, including staining by H&E.

Previously, we reported a case of a skin infection by a 40-nm papovavirus in an immunosuppressed patient [6]. In the case presented here, a viral diagnosis was suspected, but an accurate identification would have been missed without TEM. Molluscum contagiousum was considered based on location of the infection in skin and LM of a lesion, but the primarily nuclear inclusions prompted further investigation. Molluscum, a poxvirus seen in skin of immunosuppressed patients, is a virus that replicates in the cytoplasm. In this case, inclusions were observed in the nucleus by LM.

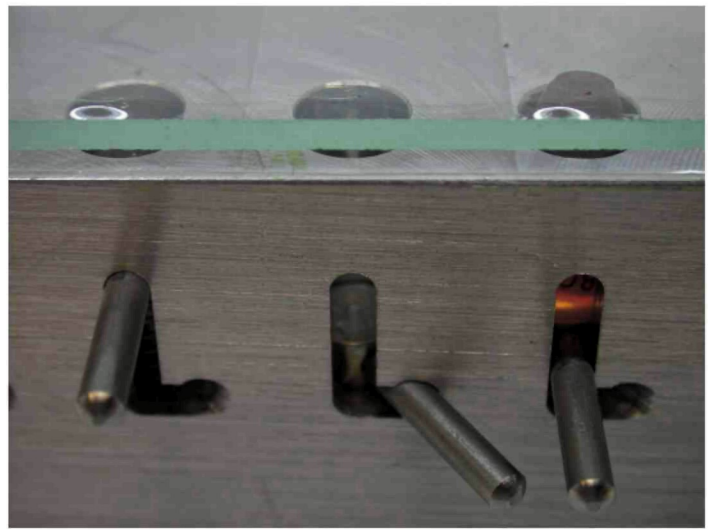

Figure 1. Close up view of aluminum apparatus for holding epoxy blocks against an acetate film under a glass plate. The spring-loaded lever can be retracted for block loading and released after the film and plate are clamped into place.

Since the only available tissue was that which had already been cut in 6-micron sections and processed by H&E staining, we removed the coverslip with xylene, scored the slide with a diamond-tipped pencil around the area of interest, and broke it to preserve some sections on the slide for additional LM if necessary. In our case, there were several sections on the slide but no other unprocessed tissue, and we wanted to save some sections for future

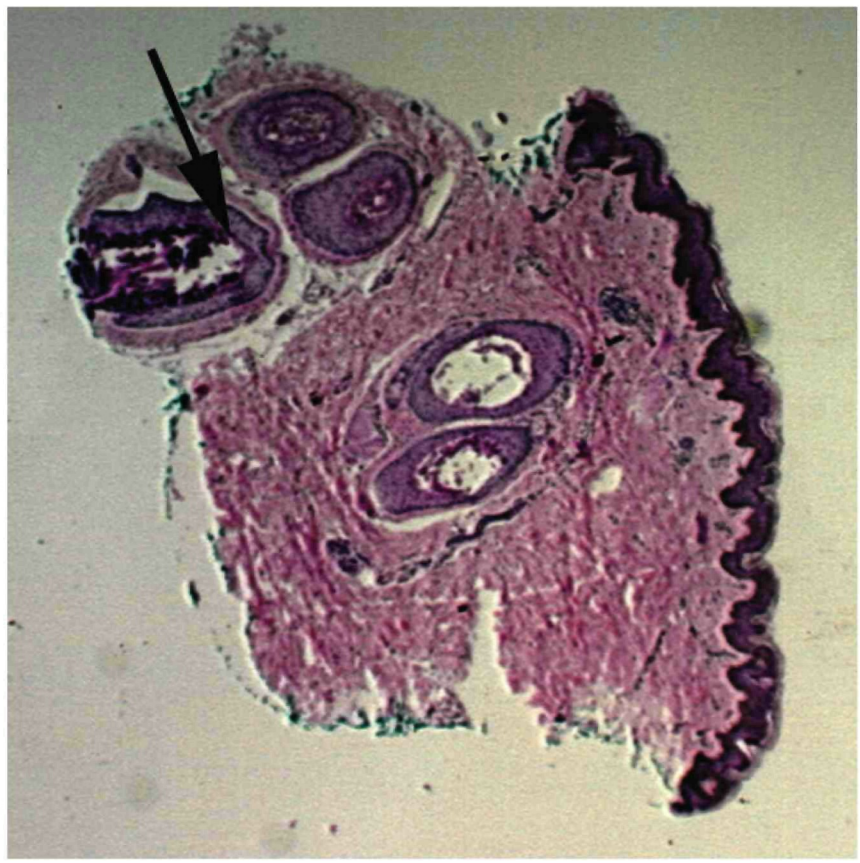

Figure 2. Light micrograph of H&E-stained skin shows epithelium (right) and hair follicles. Arrow indicates diseased hair follicle from which electron micrographs were taken.

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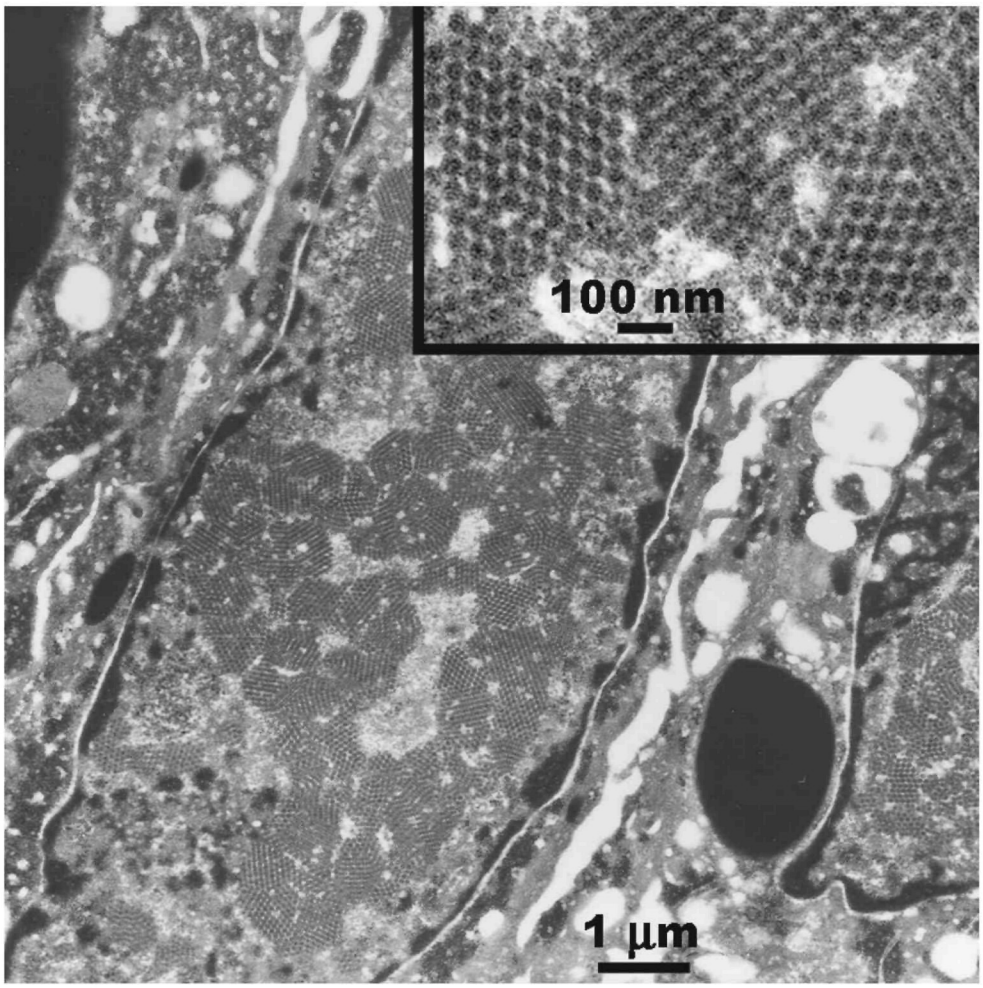

Figure 3. Low magnification electron micrograph shows nuclear inclusions of viruses. Inset shows high magnification of naked icosahedral viruses (papovaviruses) in paracrystalline arrays.

observation. We then embedded the remaining sections on one end of the slide for TEM.

The deparaffinized sections were treated *in situ* on the slide by rehydration with descending alcohols, rinsing in cacodylate buffer, fixation in 4% glutaraldehyde, post-fixation in 1% osmium, re-dehydration with a graded series of ethanols and propylene oxide, and infiltration with Poly/Bed 812. A thin layer (~1 mm) of resin (approximately 1.5 ml) was left on the slide, making sure to clean the resin thoroughly from the underneath side to prevent its sticking to the support in the oven. It was then baked overnight at 60 ^{O}C. Following polymerization the next day, the area of interest was then scored on top of the polymerized resin so as not to lose it after separation.

The glass slide and layer of resin were warmed in a beaker of water that had been heated to boiling and removed from the burner to make the resin pliable. A technique has been described whereby partially polymerized resin is peeled up from slides [2], but this necessitates that the resin removal be done at precisely the right time where it is pliable but not tacky. Dipping into just boiled water eliminates the short window of working time and renders the completely polymerized resin soft enough to bend without cracking. Separation of the two layers was initiated with a razor blade and finished by simply pulling up the pliable resin layer while warm. Occasionally, if the resin cools and begins to crack, it may have to be re-dipped into hot water to complete the separation. In our hands, the "Epon-equivalent" resins (e.g., SPI-Pon, EMBed) release from the slide easier than some of the other epoxy resins; acrylic resins were not attempted.)

The resin slab was laid flat, section side up (the surface that was against the slide), to cool. A small piece containing the tissue of interest was cut out with a razor blade and glued with the section side up onto a blank pre-polymerized epoxy block for ultrathin sectioning. The glue used was the same material as the original resin. (Note that at least one of the "non Epon-equivalent" epoxy resins may become brittle and hard to section if re-baked to attach the tissue slab to the blank block.) Since the tissue is very thin, care must be taken to place the cut-out section absolutely flat for ultrathin sectioning. We devised a holder whereby blocks containing fresh epoxy as glue and the removed section can be held tightly against a flat surface for hardening (Fig. 1). An acetate film was placed between the glass plate and the section to prevent its sticking to the glass. The apparatus was then baked overnight. No semithin sections were cut; rather, the very first and subsequent ultrathin sections were collected because the tissue was only 6 µm thick. The H&E-stained section serves as the survey semithin section (Fig. 2).

One might assume that the paraffin embedment, staining, and drying for LM plus xylene treatment of the tissue to remove the wax and coverslip would render viral structure unrecognizable. However, in the case of 40-nm naked icosahedral viruses, they remain intact enough for identification (Fig. 3). We have also shown that larger icosahedral viruses, such as adenoviruses and herpes virus nucleocapsids are identifiable after processing for LM. Other viruses, such as smaller (25-30 nm) icosahedral viruses and enveloped viruses with helical nucleocapsids or morphologically indistinct nucleocapsids may not remain as easily recognizable. However, we have identified paramyxoviruses (enveloped viruses with helical nucleocapsids) from unstained paraffin blocks of tissue that had been suspected of being virus-infected.

We conclude that this method is useful for identifying many viruses by EM, even after previous harsh treatment of paraffin embedding, deparaffinization, LM staining, and air-drying. ■

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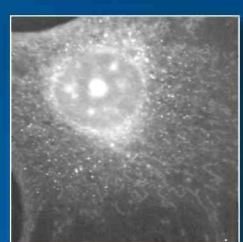
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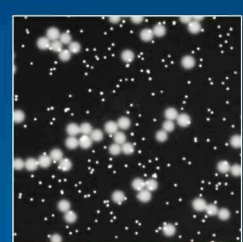
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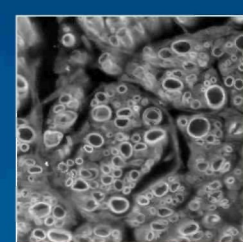
Rat sciatic nerve


Trypanosoma brucei

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