Chapter 3

Negative and Positive Staining in Transmission Electron Microscopy for Virus Diagnosis

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Additional information is available at the end of the chapter

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Abstract

Visualization of virus particles and morphological features depends on the resolution of microscopes. Transmission electron microscopy (TEM) is the starting point for obtaining the best resolution of images. Two different techniques are available and described in this paper. Firstly, negative staining of viral suspensions provides detailed information of virus particles' structure. It is a technique that can be quickly performed and is able to accommodate the highest magnifications of virus particles. Secondly, ultra-thin sections of virus-infected tissues or cell cultures, combined with a positive staining technique can provide information regarding the localization of viruses inside or around cells. These two complementary techniques for investigating the structure of a virus and its parasitic life cycle are presented in this paper.

Keywords: transmission electron microscopy, negative staining, positive staining

1. Introduction

High resolution microscopes are essential for visualizing virus particles. A transmission electron microscope (TEM) fulfils this requirement and is widely used by scientists. Unknown agents of diseases may be quickly detected by using such a microscope. A recent review on the value of electron microscopy and virus structures was presented by Zhang et al. [1].

Shortly prior to the recent recognition of mimiviruses [2], orthopoxviruses had been one of the largest virus particles, in addition to the thin and very long filoviruses. Groups of orthopox-

virus particles can be detected when inside infected cells using the highest magnification available in light microscopy. Nevertheless, an isolated virus particle of ca. 250 nm diameter remains below the resolution of standard light microscopes. With the advent of TEMs and high magnifications of over 100 000x, the structural details of virus particles could then begin to be recognized.

The first demonstration of a viral particle using a TEM was in the form of a member of the orthopoxvirus genus [3]. Following the introduction of the negative staining technique in the 1960s [4], a variety of viruses has been discovered and classified based on morphological characteristics such as symmetry, the presence or absence of the envelope or spikes and the size of these projections [5,6,7,8]. A large number of viruses of medical importance that had formerly never been described such as adenovirus, enterovirus, orthomyxovirus, reovirus and paramyxovirus were identified by TEM after isolation in cell cultures inoculated with clinical specimens [9]. Other viruses like hepatitis B and hepatitis A were detected directly by TEM in samples such as plasma and faeces, following the failure of attempting to try and isolate them in cell cultures. The initial classification of many agents was therefore based on a combination of morphology and genome structure.

Currently, more than 30 000 different viruses comprising 56 families have been identified using TEM and humans have been found to play host to 21 of the 26 families specific for vertebrates [10]. The development of other techniques, e.g., immunofluorescence, enzyme-linked immunosorbent assays (ELISA) and biological molecular methods such as polymerase chain reaction (PCR) have progressively reduced the importance of TEM in virus diagnosis and microbiology. However, compared with other diagnostic methods, TEM still benefits from its rapidity and "open view", i.e., the capability of detecting all pathogens present in a clinical specimen [11]. Therefore, TEM should be utilized as a frontline method in infectious disease emergencies and/or in suspected cases of bioterrorism [12]. Electron microscope studies were critical in identifying the aetiologic agent of severe acute respiratory syndrome (SARS), a coronavirus, during its 2002/2003 global outbreak [13].

Several methods for specimen preparation have been developed. These can be summarized into two procedures: the negative staining of vesicular content and viral suspensions, and the ultra-thin sectioning of infected tissues and cells. Both techniques have been carried out with complementary results.

2. Negative staining technique

The classic processing of biological specimens observed in a TEM needs fixation, dehydration, sectioning and a selective "staining" of cell and tissue structures. "Staining", a means of receiving coloured images, cannot be effectively used in conjunction with an electron microscope. Instead, the enhancement of structures for TEM observation is effected, usually by impregnation with heavy metal salts of plumb, tungstenium and uranium.

Some biological elements are very small and as a result, sectioning reveals aspects of its content but not about its global surface structure. The result is a bi-dimensional image. Nevertheless,

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when observed as a whole using the negative staining technique, these elements reveal a tri-dimensional image.

To prepare small biological specimens such as bacteria, viruses, phages, micoplasma, filaments and cell membranes, or even nucleic acids and protein filaments for TEM observation, the special technique of "negative contrast" was developed [4]. Instead of applying a more or less strong positive contrast of structures ("staining"), in this instance, contrast is not applied to the object but to its environment, using heavy metal salts. The electron beam can cross biological material easier than the surrounding space. The result resembles an inverted traditional TEM image (Figures 2, 4-7).The standard staining solution used today is an aqueous 1% phosphotungstic acid with pH 7.2.

Essentially, a fixed or unfixed drop of viral suspension is applied onto a formvar- or collodium-covered electron microscope grid for a few seconds; the liquid is absorbed by a filter paper, then a drop of the staining solution is applied and few seconds later also absorbed (Figure 1). After drying, the specimen is ready to be introduced into the electron beam.

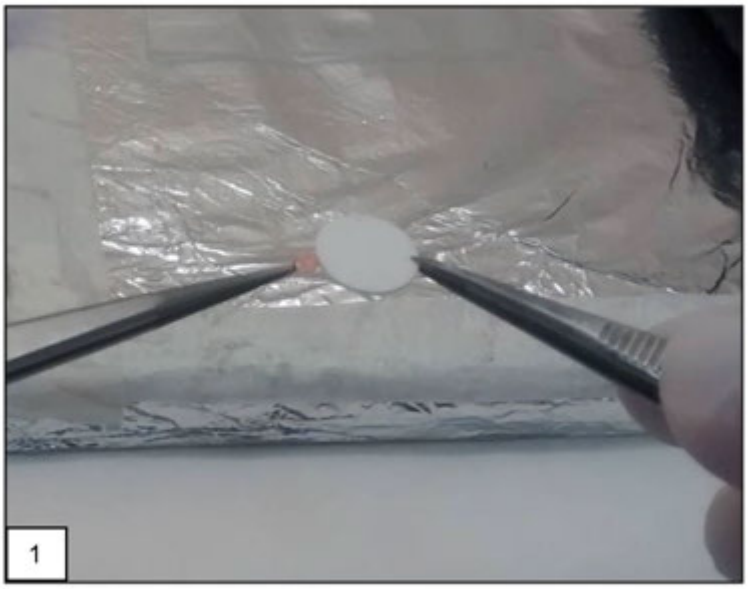

Figure 1. Negative staining procedure. Forceps, grid and white filter paper are the primary tools.

This unbelievably simple technique, at the outset rejected by the scientific community, eventually became a revolutionary approach for studying primarily viral morphology as emphasized by viral diagnostics [14]. Extremely detailed images were obtained and published, revealing substructures and macromolecules as viral antibodies and virus particle spines [5, 9, 15, 16, 17, 18, 19, 20].

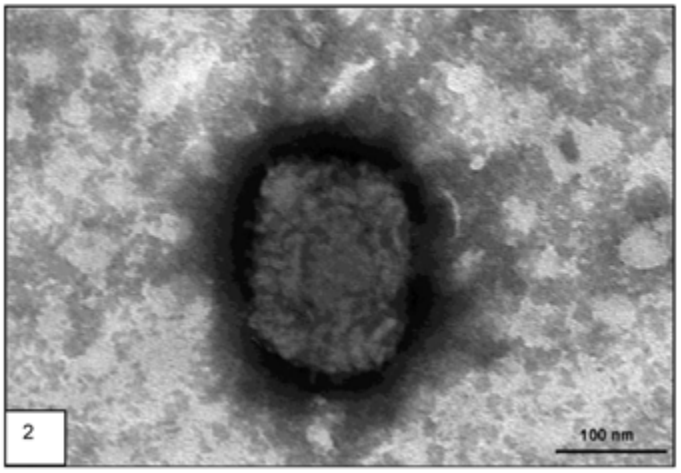

Figure 2. Orthopoxvirus particle, Brazilian Cantagalo strain (negative staining). This virus particle was isolated from a milker's hand nodule, macerated using a drop of distilled water and applied onto a collodium-covered EM-copper grid, then stained using a drop of 2% phosphotungstic acid in distilled water.

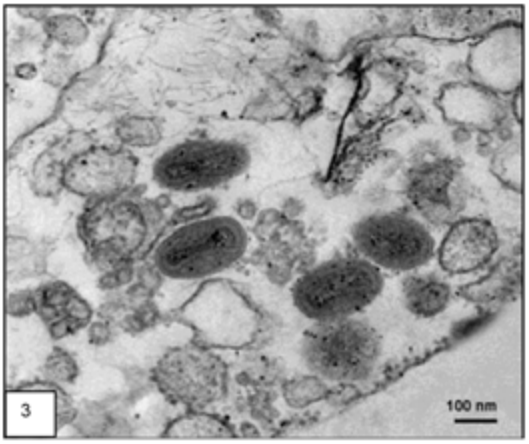

Figure 3. Ultra-thin section of a Vero cell infected with the same strain of virus demonstrated in Figure 2 (positive staining).

In order to gain additional information about small structured elements, several technical steps in sample processing were developed such as immune-electron microscopy (Figure 5), solid-phase-electron microscopy, ammonium-sulphate precipitation, gradient fraction contrasting and particle concentration by diffusion in an agarose layer [21].

Contrasting solutions show a large spectrum of possibilities. Selection must be in accordance with the pH and electrical charge of the sample, of the contrasting solution and of the EM grid

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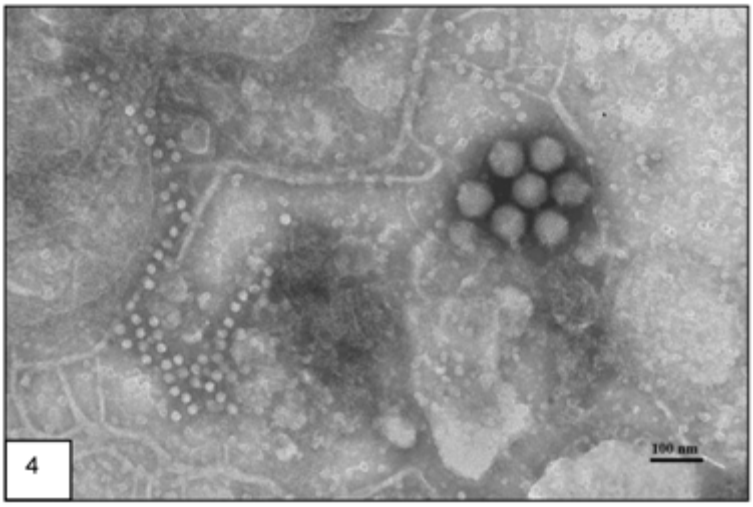

Figure 4. Group of seven adenovirus particles and numerous small adeno-associated virus particles. A drop of clarified faeces from a patient suffering from gastroenteritis was processed as described in Figure 1.

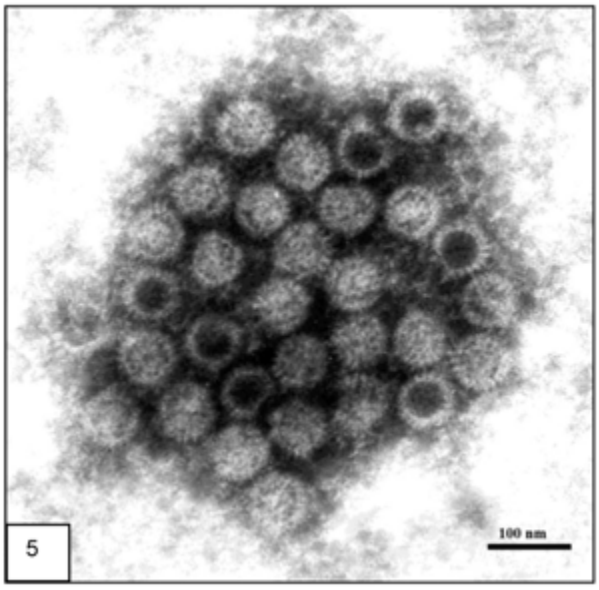

Figure 5. A group of rotavirus particles. Clarified faeces from a patient suffering from gastroenteritis was incubated with a specific antibody prior to being submitted to the negative staining procedure and processed as described in Figure 1. Both several empty and RNA-filled virus particle can be distinguished.

or support [19, 22]. Resulting precipitates and a lack of spreading of the sample are the most common inconveniences. For better spreading and adsorption of virus particles onto a formvar-carbon coated grid, polylysine (poly-L-lysine) is currently being used in our laboratory (Figures 6 to 7).

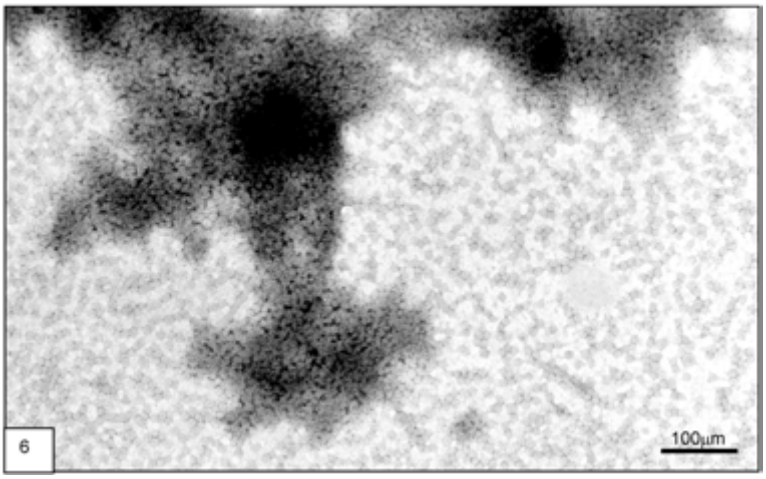

Figure 6. Hepatitis B antigen. One drop of a gradient virus suspension was applied directly onto an electron microscope grid and in sequence stained with 1% alcoholic uranyl acetate. Virus particles are joined together into very dark groups.

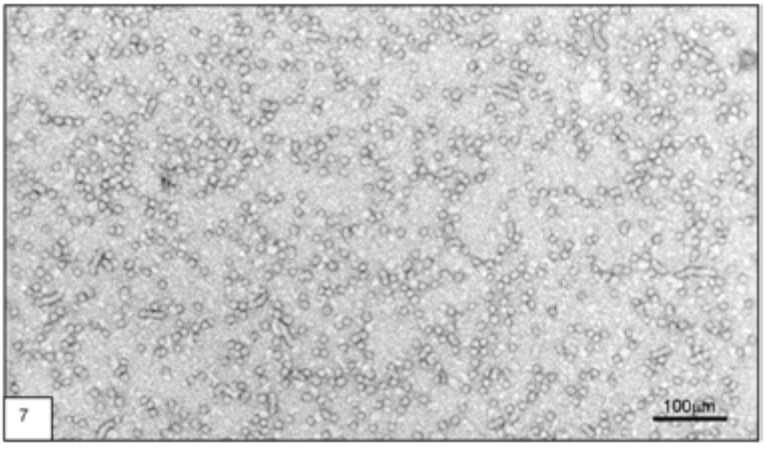

Figure 7. Hepatitis B antigen. One drop of the same virus suspension as shown in Figure 6 was applied to an electron microscope grid formerly coated with polylysine and in sequence stained with 1% alcoholic uranyl acetate. Virus particles are spread all over the grid.

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An overview of the negative staining technique development and application was presented by Biel & Gelderblom [6], Harris et al. [23] and more recently, by Schramlová et al. [24]. Benefits or deception are always surprising factors when the electron beam reveals a TEM image inside a dark room.

3. Positive staining technique

The positive staining technique has been used since the late 50s and the early 60s for enhancing the contrast of biological samples (tissues and cell structures, viruses, etc.). Using this technique, as well as negative staining, the samples are incubated in heavy metal salt solutions that react with cellular structures. Uranyl acetate [25] and lead citrate [26] are the most commonly used salts today. Grids containing ultra-thin sections of a sample are incubated for 15 minutes in uranyl acetate; this procedure should be performed in an environment protected from light. Following on, the grids are washed in distilled water and incubated in lead citrate at four to five minutes in an environment free of CO_2. NaOH tablets are used to keep the environment free from reacting with CO_2 (Figure 8). At the end of the procedure the grids are washed in distilled water, air dried and observed with a TEM [27, 28].

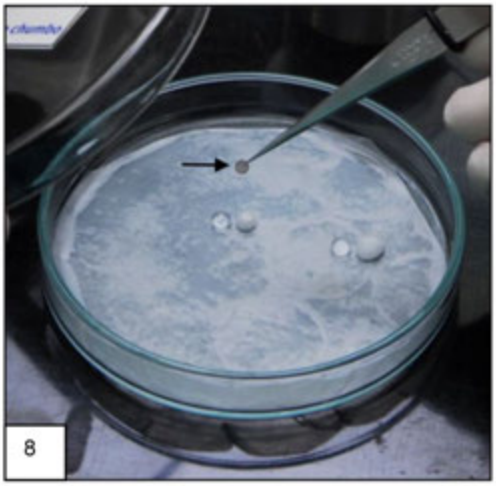

Figure 8. Positive staining. The grid held with a forceps contains ultra-thin sections of a sample (arrow). In the Petri dish, there are two drops of the staining solution beside two tablets of NaOH (Barreto-Vieira et al. 2010).

3.1. Specimen preparation

Cells. Monolayers were fixed in 1% glutaraldehyde in a cacodylate buffer (0.2 M, pH 7.2) and post-fixed with 1% buffered osmium tetroxide, dehydrated in acetone, embedded in epoxy resin and polymerized at 60^0C for three days [28, 29]. The blocks were cut into ultra-thin

sections 50 to 70 nm thick using a diamond knife adapted to an ultramicrotome (Figure 9). The sections were placed onto copper grids and stained with uranyl acetate and lead citrate and observed in a TEM (Figure 10).

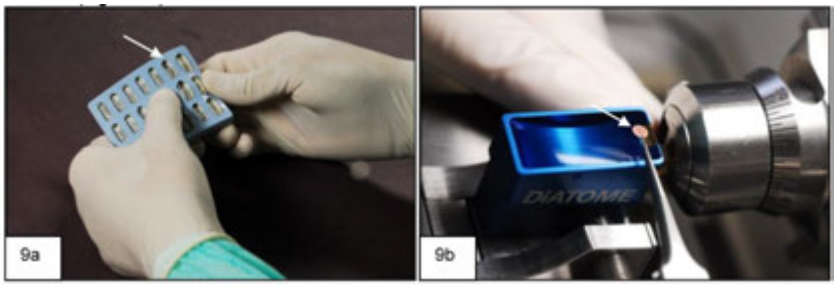

Figure 9. Ultramicrotomy: **a)** silicone support filled with resin blocks (arrow) containing fragments of tissues; **b)** ultrathin sectioning using a diamond knife and a copper grid (arrow) to collect sections.

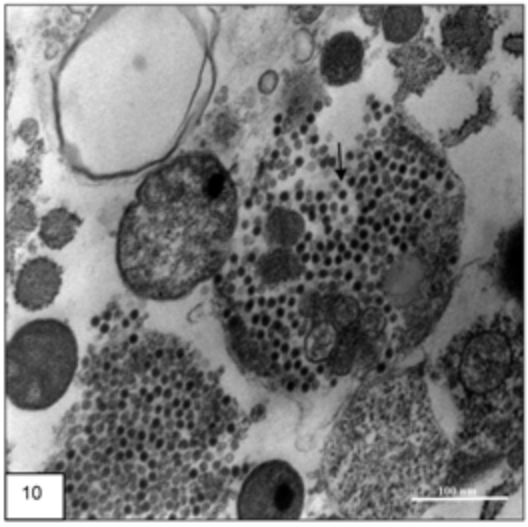

Figure 10. Dengue virus particles (arrow) isolated in the *Aedes albopictus* mosquito cell line (C6/36) from the serum of infected BALB/c mice. Positive staining using lead citrate (arrow) emphasizes viral RNA, while cytoplasmic proteins are emphasized using uranyl acetate.

Fragments of tissues. Glutaraldehyde at 2% in a cacodylate buffer (0.2M, pH 7.2) was routinely used to fix tissue fragments, then post-fixed by immersion in a 1% buffered osmium tetroxide, dehydrated in acetone, embedded in epoxy resin and polymerized at 60^0C for three days [28, 29]. Semi-thin 0.5 μm thick sections were cut using a diamond knife. These sections were stained with a methylene blue/azure II solution [30] and observed using a photonic micro-

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scope. Ultra-thin sections were placed onto copper grids and stained with uranyl acetate and lead citrate and observed in a TEM.

Tissue fragments from experimentally infected animals. In our experiments, the infected animals (mice) were peritoneally anaesthetized and fixed by perfusion with a 4% paraformaldeyde in a sodium phosphate buffer (0.2M, pH 7.2) for 30 min. The tissues were carefully collected in sequence and the fragments post-fixed by immersion in 2% glutaraldehyde in cacodylate buffer (0.2M, pH 7.2), post-fixed with 1% buffered osmium tetroxide, dehydrated in acetone, embedded in epoxy resin and polymerized at 60^0C for three days [28, 29]. Ultra-thin sections were obtained and processed as described above (Figure 11).

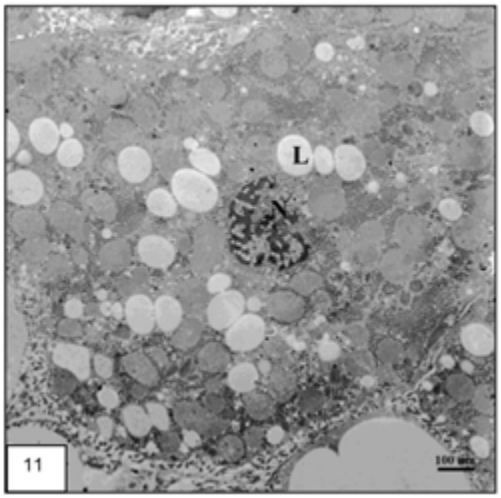

Figure 11. Hepatic tissue of BALB/c mice infected with the dengue virus. Note the numerous lipidic inclusions (L) and a pyknotic nucleus (N). Positive staining emphasizes the chromatin of the hepatocyte nucleus using lead citrate and cytoplasmic proteins using uranyl acetate.

4. Advantages and disadvantages of the TEM

Virus diagnosis via TEM, when compared to other techniques such as immunofluorescence, PCR and ELISA have both advantages and disadvantages.

*Advantages:***1)** TEM is the only technique that allows for direct visualization of an aetiologic agent of very small diameter, e.g., viruses; all other diagnostic techniques show indirect results; **2)** samples can be processed and analysed quickly. Following simple and rapid negative staining, TEM "open view" allows for rapid morphological identification and a differential diagnosis of various infectious agents present in a specimen. Likely, intact virions observed with TEM may be indicative of an infectious virus, whereas the detection of antigens or nucleic

acid may not always indicate the presence of viable infectious virus particles [31]; **3)** TEM is valuable for differential diagnoses, for example, in patients with vesicular dermatitis, so as to exclude smallpox; **4)** TEM assists in elucidating unknown aetiologic agents in outbreaks, epidemics or pandemics, since it is not necessary to use specific reagents. The causative agents of the recent SARS pandemic and the outbreak of human monkey pox in the USA were quickly identified using TEM [13].

*Disadvantages:***1)** TEM is of low diagnostic sensitivity compared to other diagnostic methods. It requires a high concentration of virus particles (>10^5 particles per mL), both in suspensions and in tissues or cells; **2)** the evaluation of samples cannot be automated with TEM and should be performed by an experienced professional; **3)** TEM is not an appropriate method for screening a large number of samples as in a viral outbreak, due to the high cost of TEM maintenance and the availability of trained personal.

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