Virus detection by transmission electron microscopy: Still useful for diagnosis and a plus for biosafety

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Summary
Transmission electron microscopy (TEM) is the only imaging technique allowing the direct visualization of viruses, due to its nanometer-scale resolution. Between the 1960s and 1990s, TEM contributed to the discovery of many types of viruses and served as a diagnostic tool for identifying viruses directly in biological samples, either in suspension or in sections of tissues or mammalian cells grown in vitro in contact with clinical samples. The diagnosis of viral infections improved considerably during the 1990s, with the advent of highly sensitive techniques, such as enzyme-linked immunosorbent assay (ELISA) and PCR, rendering TEM obsolete for this purpose. However, the last 20 years have demonstrated the utility of this technique in particular situations, due to its “catch-all” nature, making diagnosis possible through visualization of the virus, without the need of prior assumptions about the infectious agent sought. Thus, in several major outbreaks in which molecular techniques failed to identify the infectious agent, TEM provided the answer. TEM is also still occasionally used in routine diagnosis to characterize infections not diagnosed by molecular assays. It is also used to check the microbiological safety of biological products. Many biopharmaceuticals are produced in animal cells that might contain little-known, difficult-to-detect viruses. In this context, the “catch-all” properties of TEM make it possible to document the presence of viruses or virus-like particles in these products.

KEYWORDS
electron microscopy, viral diagnosis, viral safety, virus

1 | HISTORY

The first transmission electron microscope was developed in the early 1930s by Ernst Ruska with his PhD supervisor, Max Knoll.1,2 This microscope had a much higher resolution than any of the light microscopes available at the time and promised to revolutionize many aspects of science, including cell biology and virology. Ernst Ruska was a physicist (1986 Nobel prize winner in physics), but his younger brother, Helmut Ruska, who had trained in medicine, rapidly recognized the potential of this microscope for investigating the nature of viruses.3 In the early 1940s, viruses were classified according to their hosts and the clinical symptoms they caused. Despite the lack of established methods of biological sample preparation for transmission electron microscopy (TEM) at this time, Helmut Ruska was able to characterize the morphology of several viruses and he developed a rough viral classification based on the size and shape of the viral particles.4 TEM was rapidly adopted for its first major use in clinical virology: the differential diagnosis of smallpox, caused by the variola virus.
from the poxvirus family, and chickenpox, caused by the varicella-zoster virus of the herpes family, based on investigations of fluid samples from the vesicles on the patients' skin. The chickenpox virus appeared to be spherical and 140 to 150 nm in diameter, with a central body, a structure clearly different from that of the much larger, brick-shaped smallpox virus.

1.1 Role of the TEM in the discovery of viruses and routine diagnosis until the 1990s

The introduction of negative staining, based on aqueous suspensions of biological particles deposited on carbon-coated grids and stained with heavy metals salts (such as uranyl acetate or phosphotungstic acid), improved the observation of viral particles, paving the way for the widespread use of TEM in basic virology and for the rapid diagnosis of viral infection (Figure 1A-D). Negative staining clearly distinguishes the viral particle from the background and provides precise morphological information (concerning symmetry and the presence or absence of an envelope, for example), facilitating the specific identification of viruses, or at least their classification into morphologically similar groups. The use of TEM for viral studies peaked in the 1970s and 1980s, when this technique contributed to the discovery of many clinically important viruses, such as adenovirus, entero-, paramyxovirus, and reovirus, all of which were isolated and observed after propagation in cell cultures in vitro. Differences in virus size and fine structure were used as criteria for a more precise classification. However, TEM initially failed to detect agents for other diseases, such as hepatitis and gastroenteritis, because the causal viruses could not be propagated in cell cultures in vitro. Nevertheless, the application of TEM to

FIGURE 1 Diagnosis of viral infections by transmission electron microscopy (TEM). Panels A to C show negative-staining TEM images of viruses present in human biological samples examined for routine diagnosis. Panels A and B illustrate rapid differential morphological diagnosis comparing a herpesvirus (A) and a parapoxvirus (B) in fluid recovered from skin vesicles. Panels C and D illustrate rapid differential morphological diagnosis for a rotavirus (C) and an adenovirus (D) in feces. Panel E and F show ultrathin sections of human tissue or cells (with high magnification in insets): E, parapoxvirus (Orf virus) infection on a human skin biopsy specimen; F, polyomavirus (BK virus) infection in cells obtained from a urine sample from a transplant recipient. The scale bars correspond to 100 nm (in A,C,D), 200 nm (in B,E) or 500 nm (F)
“dirty” clinical samples, such as plasma, urine and feces in the 1970s constituted a major breakthrough for studies of these viruses. The etiologic agents of hepatitis B and A were detected in plasma and stool samples, respectively. The BK virus, a polyomavirus, was identified for the first time in the urine of patients undergoing renal transplantation. Rotaviruses were also identified by TEM as the main cause of epidemic gastroenteritis in humans and animals. However, many other viruses were also found to cause gastroenteritis. The first of these viruses was the Norwalk virus, identified during an outbreak of gastroenteritis in Norwalk, Ohio, USA. Viruses with a similar morphology were subsequently discovered elsewhere and called “Norwalk-like” viruses, to reflect the similarity of their appearance on TEM, before being officially renamed “noroviruses.” Other viruses from the adenovirus, astrovirus, and calicivirus families were also identified in the stool samples of children with gastroenteritis. TEM was, thus, widely used on negatively stained samples for routine diagnosis, as a rapid, “catch-all” method for distinguishing between the diverse viruses potentially implicated in human gastroenteritis, providing a diagnosis within 15 minutes of the arrival of the sample in the laboratory.

Although more time-consuming, due to the need to embed a sample in resin and cut ultrathin sections with an ultramicrotome, the TEM has also proved useful in medical virology, in searches for viruses in tissues. Panels E and F in Figure 1 illustrate a parapoxvirus (Orf virus) visualized in a skin biopsy specimen from a patient with a severe finger ulcer and a polyomavirus (the BK virus) in cells collected from the urine of a renal transplant patient with nephropathy, respectively.

1.2 Declining the use of TEM for routine viral diagnosis since the 1990s

Major changes in the diagnosis of viral infections occurred in the 1990s, with the advent of more sensitive molecular techniques, such as enzyme-linked immunosorbent assays (ELISAs) and polymerase chain
reaction (PCR) in particular. Molecular techniques, with their advantages of greater sensitivity and the capacity to process large numbers of samples easily, replaced TEM in many areas of virological diagnosis. This was the case, in particular, for the diagnosis of viral gastroenteritis, for which molecular techniques capable of identifying most of the virus families involved in human gastroenteritis have been established.24-27 A similar shift in practice occurred in veterinary medicine, with ELISAs and PCR progressively replacing TEM for the routine diagnosis of viral infections.28-31 In human medicine, the use of TEM to differentiate between smallpox virus and the other viruses present in the fluids of cutaneous vesicles is no longer required, since the successful eradication of the variola virus in 1980 thanks to a worldwide vaccination program.32 It has been argued that TEM remains potentially useful for this application in a context of bioterrorism.33,34 However, the risk of smallpox reappearing is extremely small, and even in the unlikely event of this happening, molecular techniques would undoubtedly outperform TEM for this diagnosis. Consequently, the number of laboratories making use of TEM for diagnostic purposes has decreased considerably.

2 CURRENT ROLE OF TEM IN THE DIAGNOSIS OF VIRAL INFECTION

TEM remains very useful for resolving certain diagnostic problems in medical virology, as clearly illustrated on several remarkable occasions over the last 20 years. In most of these cases, TEM was not used to characterize the virus directly in the patient sample, but after isolation of the virus from the clinical sample by propagation in a cell culture in vitro. During an outbreak of fatal respiratory disease in horses and influenza-like illness in humans in Australia in 1995, TEM proved essential for the identification of a previously unknown virus, the Hendra virus, recognized as a member of the Paramyxoviridae family on the basis of its ultrastructure.35 A related virus, the Nipah virus, was also first identified by TEM on cerebrospinal fluid, during an outbreak of encephalitis in Malaysia and Singapore in 1999 in men who had been exposed to pigs.36,37 The etiology of the severe acute respiratory syndrome (SARS) pandemic in Hong Kong and Southern China in 2003 was also initially determined by TEM. The causal virus was isolated in several laboratories around the world, by inoculating cell cultures with respiratory specimens, leading to the identification of a coronavirus on ultrathin TEM sections of these cells.38,39 An outbreak of an unidentified rash in humans, associated with an illness in prairie dogs, occurred in the United States in 2003. TEM revealed the presence of a poxvirus in a cell culture isolate, and this virus was later identified as a monkeypox virus.40 The etiologic agent responsible for an acute severe fever with thrombocytopenia syndrome (SFTS) in six provinces of China in 2011 was diagnosed through the use of TEM to investigate cells cultured in the presence of blood samples from patients with the disease. TEM revealed the presence of virions with...
the characteristic morphological features of a bunyavirus. RNA sequence analysis revealed that this virus was, indeed, a new member of the genus Phlebovirus in the Bunyaviridae family.

TEM has also been successfully used to elucidate unexplained symptoms in small transmission clusters, and even in isolated cases. It was used, for example, in cases of graft dysfunction, fever, and altered mental status in transplant recipients in the United States in 2006. A virus from the Arenaviridae family was observed in ultrathin sections of Vero cells grown in the presence of a cerebrospinal fluid sample from one of these patients. PCR was then used to characterize the virus in more detail. It was identified as lymphocytic choriomeningitis virus (LCMV), an arenavirus transmitted by rodents. TEM was also used to identify West Nile virus in a skin specimen from a patient with an enigmatic hemorrhagic fever in the United States in 2006. The virus was identified by visualization in ultrathin sections of Vero cells grown in the presence of a homogenate of the skin biopsy specimen. Several cases of bunyavirus infection have also been diagnosed by TEM in patients bitten by mosquitoes or ticks in the United States and presenting with encephalitis or fever and fatigue syndrome. In Austria, in 2014, TEM identified picornaviruses in the urine samples of a group of neonates, all born at the same hospital and suffering from a sepsis-like illness, after negative results were obtained from the initial microbiological tests. TEM also made possible the direct detection, in the brain tissues, of a polyomavirus (the JC virus) responsible for cases of fatal progressive multifocal leukoencephalopathy, in the absence of viral DNA in the cerebrospinal fluid. TEM is still occasionally useful in cases of human gastroenteritis, for the identification of new subtypes of causal viruses such as adenovirus, picornavirus, or calicivirus not recognized by molecular techniques.

Thus, in recent years, the role of TEM in the diagnosis of viral infections has shifted from routine use to an initial screening test for the identification of unknown infectious agents in particular outbreaks or viral transmission clusters. In such investigations, the underlying “catch-all” principle of this technique is a major advantage for the recognition of an unknown agent, as viruses from various families have different morphological appearances, which are used as the basis of initial virus identification by TEM. This method allows an “open view,” sometimes revealing unexpected infectious agents, while molecular methods require previous knowledge of the virus to be tested. TEM has also the advantage of being able to potentially identify double or multiple infections caused by more than one virus, which could be missed by molecular or antigen tests. Moreover, the nature of the samples to be analyzed can be diverse, from body fluids or biopsies analyzed directly or after cell culture.

In some cases, TEM has also been used to confirm a diagnosis previously established with molecular techniques. Figure 3 illustrates

FIGURE 5 Detection of retroviruses budding at the cell surface of rodent cells used for the production of biological products. These four ultrathin sections of cells examined by transmission electron microscopy (TEM) show C-type retroviral particles budding at the plasma membrane and released into the extracellular medium. The viruses indicated by the arrows are shown at high magnification in the inset. Scale bars represent 200 nm (A,D) or 500 nm (B,C)
the detection of a reovirus strain in MRC5 cells cultured in the presence of urine and throat swab specimens from two children with unexplained neurologic symptoms of encephalitis. In this case, TEM confirmed the results of molecular techniques and contributed to the identification of a previously unknown reovirus strain as an etiologic agent of encephalitis.55

In veterinary medicine, TEM has frequently proved useful for identification of the virus responsible for particular outbreaks of disease.56-65 Immuno-TEM with serum from convalescent domestic or wild animals has proved useful for the detection of unknown etiological agents, in situations in which alternative diagnostic methods are unsuccessful due to the lack of immunological reagents and primers.66

3 | TEM IN VIRAL SAFETY

TEM is also considered an important method for the assessment of viral safety in biopharmaceutical products. TEM is recommended in the guidelines of the US Food and Drug Administration (FDA) and European Medicines Agency (EMEA), which also specify the materials to be tested, including cell lines, culture supernatants, and fermenter bulk harvests.67,68 Although time-consuming and of limited sensitivity, TEM is recommended, in particular, for its “catch-all” properties, as a complementary approach to in vitro assays and molecular techniques. Current regulations for the use of animal-derived components in biopharmaceutical products stipulate a number of source-testing and manufacturing measures to be implemented, to minimize the potential risk of viral contamination. The in vitro assays for virus detection use a selection of cell lines with history of successful use for the detection of a wide range of potential virus contaminants.69 They can be used in the testing of culture medium in cell banks, and for the testing of raw materials, such as the bovine serum or other animal-derived growth factors used in cell culture. However, infections can occur in cultured cells without a cytopathic effect, and such infections may be missed. Various other molecular assays can be used such as fluorescent product-enhanced reverse transcription (FPERT),70 which is specific for retroviruses, or PCR-based tests if specific risks have been identified during risk evaluation. By combining all these methods, it is possible to cover a broad spectrum of potential contaminants, although it is never possible to provide a 100% guarantee that no unwanted agents are present. In this context, TEM constitutes an additional check in the viral safety testing of biological products as it can document the possible presence of viruses or virus-like particles in master cell banks or fermenter bulk harvests.

Rodent cell lines are widely used for the manufacture of recombinant proteins for pharmaceutical use in humans such as monoclonal antibodies, vaccines, and viral vectors for gene therapy. These cell lines have long been known to contain retroviral elements, because the rodent genome contains many copies of endogenous retrovirus-like sequences.71 Most of the viral particles produced by these cells such as intracytoplasmic or intracisternal A-type particles are defective and noninfectious (Figure 4). However, other particles such as C-type particles bud at the cell surface and may infect nonrodent cells72 (Figure 5). Some murine retroviruses have been shown to be tumorigenic in primates,73 and cases of leukemia have been reported in children with severe combined immunodeficiency treated by gene therapy involving the use of murine retroviral vectors.74 All these elements demonstrate the relevance of tracking the presence of retroviruses in biological products derived from rodent cells. Reverse transcriptase assays performed on bulk harvests are often hampered by high background levels due to cell-derived DNA polymerases.75 TEM may, therefore, help to document the presence of retrovirus-like particles in these bulk harvests (Figure 6). TEM can also be used to gauge the concentration of viral particles to validate the clearance of retroviruses or any other virus suspected to be present in the master cell bank. Fortunately, the endogenous retroviruses present in the Chinese hamster ovary (CHO) cell line, the main rodent cell line used to produce biological products in the biotech industry, have been shown to be noninfectious.76 Testing requirements are now lower for this well-characterized cell line than for other cell lines with which experience is more limited. Nevertheless,
novel cell substrates, including insect cell lines in particular, are now being introduced into the biotech industry. Their use will carry new concerns about unknown viruses for which there is a potential risk of contamination, and TEM will undoubtedly be useful for documenting the presence of viruses or virus-like particles in these cells and the products derived from them.

4  |  CONCLUDING REMARKS

In conclusion, although TEM is sometimes seen as a somewhat "old-fashioned" technique, it still has an important role to play in virus detection. It is particularly useful for identifying unknown agents involved in particular outbreaks or transmission clusters. In routine diagnosis, it may be useful to confirm or even, in some cases, to guide the diagnosis of a viral infection. TEM can also be used to check the viral safety of biopharmaceutical products. This technique has several disadvantages, such as the cost of electron microscopes and their maintenance, the need for well-trained microscopists, and time-consuming analysis, particularly if the samples must be embedded in resin for the cutting of ultrathin sections. However, all the techniques available have benefits and disadvantages, and their complementary natures mean that there are advantages to be gained by using them in combination. In this respect, the principal advantage of TEM is its ability to provide an image of the virus, providing additional confidence in the result.

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CONFLICT OF INTEREST

The authors have no competing interest.

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