Electron Microscopy for Rapid Diagnosis of Infectious Agents in Emergent Situations1

Paul R. Hazelton* and Hans R. Gelderblom†

Diagnostic electron microscopy has two advantages over enzyme-linked immunosorbent assay and nucleic acid amplification tests. After a simple and fast negative stain preparation, the undirected, "open view" of electron microscopy allows rapid morphologic identification and differential diagnosis of different agents contained in the specimen. Details for efficient sample collection, preparation, and particle enrichment are given. Applications of diagnostic electron microscopy in clinically or epidemiologically critical situations as well as in bioterrorist events are discussed. Electron microscopy can be applied to many body samples and can also hasten routine cell culture diagnosis. To exploit the potential of diagnostic electron microscopy fully, it should be quality controlled, applied as a frontline method, and be coordinated and run in parallel with other diagnostic techniques.

In late September 2001, a letter containing spores of Bacillus anthracis arrived at a publishing house in Palm Beach, Florida, and resulted in the death of one employee from inhalation anthrax. Over the next 6 weeks, similar letters were delivered to television news centers in New York City and government offices in Washington, D.C. Ultimately >32,000 suspected exposures and five deaths were recorded in the United States. The collateral spread of exposure to spores was a sobering reminder of the bioterrorism attack scenario hypothesized by O’Toole (1).

Today, technology allows genetic engineering of potentially devastating agents such as modified ectromelia virus (2), the weaponizing variola virus (former USSR) (3), the long distance dispersal of yellow fever–infested mosquitos (United States) (4), and the weaponizing of anthrax spores by many nations. The ease with which the recent anthrax attacks were delivered indicates that unsophisticated methods are still effective. Thus, the most potent defenses remain rapid identification of the event and agent, treatment of the victims, and containment of infection. Successful outbreak management depends on early recognition of a suspected infectious case by the primary care physician and obtaining an accurate, timely laboratory diagnosis. An unexpected temporal or geographic cluster of illness of apparently infectious nature or an unusual age distribution of pneumonia with respiratory failure, intradermal hemorrhage, or chickenpox-like illnesses may indicate infection caused by a novel agent or a bioterrorist act. Similarly, the sudden appearance of vesicular lesions or respiratory illness in farm animals may be evidence of an emerging disease, a possible zoonosis, or an agriterrorist act. While recent studies suggest that healthcare systems are ill prepared to treat victims and contain the spread of an infectious agent (5), the performance of physicians, epidemiologists, and diagnostic specialists in identifying outbreak-associated agents as diverse as Nipah virus (6) and gastroenteric agents (7) indicate that identification of an outbreak and its associated agent may be done rapidly and successfully.

Electron microscopic diagnosis is uniquely suited for rapid identification of infectious agents. A specimen can be ready for examination and an experienced virologist or technologist can identify, by electron microscopy, a viral pathogen morphologically within 10 minutes of arrival in the electron microscopy laboratory (8). We describe the role of transmission electron microscopy in viral diagnosis and outbreak management; methods for specimen collection, preparation, and examination; laboratory safety and quality control; and the differential morphologic diagnosis of infectious agents. In addition, an online appendix lists facilities that provide electron microscopic diagnostic support (available from: URL: http://www.cdc.gov/nicod/EID/vol9no3/02-0327-app.htm).

Role of Electron Microscopy in Virus Identification

The first electron micrograph of poxvirus was published in 1938. In 1941, immunologic procedures were first used in electron microscopic studies of tobacco mosaic virus (9), and electron microscopy was introduced successfully in the differential diagnosis of smallpox and chickenpox infections in the late 1940s (10,11). With the introduction of negative staining in the late 1950s (12) and the wider availability of electron microscopes, electron microscopy (as a catchall method) became essential in characterizing many new isolates detected in diagnostic cell cultures and clinical samples, e.g., stool, urine, and biopsied specimens (7,13–16). Pattern recognition, i.e., information on size and particle morphology, leads to rapid identification of infectious agents. The initial classification of many agents was therefore based on a combination of morphology and genome structure. Currently, >30,000 different viruses comprising 56 separate families have been identified, and humans have been found to host 21 of the 26 families specific for vertebrates (17). The distinct morphology of members of

*University of Manitoba, Winnipeg, MB, Canada; and †Robert Koch-Institut, Berlin, Germany

1 Both authors contributed equally to this review.
different viral families usually allows an agent to be assigned to a particular family. This morpho-diagnosis, combined with clinical information is, in most cases, sufficient to permit a provisional diagnosis or rule out a more serious infection and to initiate treatment and containment protocols without waiting for other test results.

Because electron microscopy is not suitable for screening large numbers of samples, many alternate immunologic and molecular methods have been developed on the basis of nucleic acid amplification techniques. While immunologic tests have almost unlimited throughput, the high specificity of these assays may result in failure to identify etiologic agents with different antigenic determinants. Further, reagents may not currently exist that would permit complete immunologic testing (18,19). Even when an immunologic test is appropriate for the etiologic agent, the sensitivity may only equal that of electron microscopy (20,21). Nucleic acid amplification techniques have similar limitations. They are more sensitive but are only capable of identifying the presence of genomic material for previously identified agents. Although primers exist that will permit amplification of most enteroviruses (22,23), few multiplex systems can identify all genotypes and serotypes within, or between, the different families of viruses that infect humans (22,24,25). Further, mutations in the primer target region may negate the effectiveness of primers. Because nucleic acid amplification techniques will not identify subviral components such as empty virions, which may be produced late in an infection, some studies suggest that their practical level of sensitivity does not always exceed that of electron microscopy (19,25,26). Because this modern armament has taken over most routine diagnostics, with the exception of gastroenteric viral infections, electron microscopy may be concentrated on infectious disease emergencies. The “open view” of electron microscopic testing allows an unbiased, rapid detection of viruses and other agents if sufficiently high particle concentrations exist (Figure 1). Because of this capability, electron microscopic testing must be a frontline method, applied either to samples directly from a suspected lesion, bodily fluids, or biopsies after cell-culture augmentation of a cultivable agent or from letters and environmental samples.

**Specimen Collection**

Successful investigation of any outbreak or novel case starts with specimen collection. Insufficient, improper, or inadequate sampling may delay or prevent identification of a causative agent. Sufficient sampling requires identification of, and sampling from, all areas where infection may have been established. Fecal samples are ideal for investigating gastroenteric episodes, as are lesion fluids or smears from skin lesions of possible viral origin.

A major cause of insufficient sampling can be failure to collect acute-phase sera from affected case-patients and more importantly, their contacts, who might well be asymptomatic. First, the existence of a blood-borne pathogen may not be evident when examining unexplained cases, as demonstrated by the difficulty identifying HIV (27) and hepatitis C virus infections, and associating human parvovirus B-19 with Fifth disease (16). Second, acute-phase sera are essential for demonstrating seroconversion to a suspected agent. Third, clinical symptoms may be caused by an immune response to an infection that has resolved by the time they appear. However, specimens from apparently uninfected contacts of patients with acute cases may contain the agent involved (16). Convalescent-phase sera collected from case-patients 4–6 weeks after onset of illness are also powerful diagnostic reagents. If no agent has
been identified by standard virus detection procedures (e.g.,
electron microscopy, tissue culture, immunoassay, or nucleic
acid amplification techniques), these serum samples may be used
to detect the causative agent (28), while matched acute:convales-
cent-phase serum pairs collected at least 2 weeks apart may be
used to demonstrate a significant rise in specific antibody among
cases by immuno-electron microscopy (Figure 2) (7). Infectious
agents may also be identified in cerebrospinal fluid, lesion
crusts, nasopharyngeal washes, saliva, tears, urine, and biop-
sied tissue specimens (29). However, low viral load, sampling
difficulties, or both may reduce the effectiveness of rapid elec-
tron microscopic diagnosis on these later types of specimens
without initial tissue culture amplification, as observed in
Nipah virus studies (6).

Safety concerns, miscommunication between infectious
diseases specialists and staff who collect samples, or inade-
quate training may result in improper sample collection.
Although swab samples placed into viral transport media may
allow nucleic acid amplification techniques or culture of non-
fastidious agents to be carried out, such specimens are not very
conducive to successful rapid electron microscopy diagnosis of
lesion exudates because of dilution effects and interfering com-
ponents. Several effective ways of collecting lesion fluids exist
(8). A method readily available to the physician or in a hospital
ward is collection into the barrel of a 26-gauge needle attached
to a tuberculin syringe. A fresh lesion is unroofed or the
beveled surface of the needle is placed against the base of an
open lesion, and fluid is aspirated into the barrel. After capping,
the sample may be transported directly for rapid electron
microscope diagnosis (Figure 3A). Alternatively, coated elec-
tron microscope specimen grids may be lightly touched direct-
ly to the vesicle fluid, lesion base, or both; allowed to air dry;
and transported directly for examination (direct touch prepara-
tion) (Figure 3B). Because repreparing the sample with direct
touch preparations may not be possible, at least two grids
should be obtained when the specimen is collected. For safety
and containment of hazardous infectious materials, the syringe
or grid should be placed in a rigid sterile container, e.g., coni-
cal 15-mL centrifuge tube or Beem capsule (Beem Co., Bronx,
NY), sealed with Parafilm (American National Can Co.,
Greenwich, CT), and the outside of the tube washed with 0.5%
sodium hypochlorite (10% household bleach) before transport
(Figure 3). Safety regulations usually require further packaging
of the sample inside a second container.

In the late 1940s, direct touch preparations from skin
lesions were prepared in North Africa and sent to Toronto,
Canada, where they were examined successfully for smallpox
virus for up to 4 months after collection (11). In another com-
parative study in Winnipeg, Canada, which used matched
lesions, we observed an average increase of 10.2:1 in the num-
ber of virions visualized by direct touch as opposed to needle
aspirate preparations, and the ratio was >1.0 in 92% of total
cases examined (n=12; p<0.02; [Wilcoxon signed-rank test]).
We observed no difference in the number of positive identifica-
tions or homogeneity of virion distribution on the grid between
these two methods. Lesion smears on glass slides may also be
used effectively for both electron microscopy and immunoflu-
orescent microscopy examination (Figure 3C). Smears, i.e.,
dried down vesicle fluids, are especially effective when
syringes and electron microscopic grids are not available. Both
direct touch and smear preparations are useful when specimens
must be transported some distance for electron microscopic
examination.

The collection of lesion exudates as swab samples placed
in viral transport medium is less effective. A change in speci-
men collection protocols in 1995, from direct touch/lesion aspi-
rates to swab specimens in transport medium, has resulted in a
decline in successful identification of virions in lesion speci-
mens in Winnipeg from 62% to 75% to approximately 10%
(Hazelton, unpub. data). While complete fecal samples are
preferable, collecting rectal swab samples for diagnosis of gas-

Figure 2. Association of human parvovirus B-19 with erythema infectio-
sum by immuno-electron microscopy. A. Airfuge EM-90 rotor (Beckman,
Palo Alto, CA) preparation of human serum prospectively collected at
time of contact with case of erythema infectiosum. Erythema infectio-
sum-like rash developed 1 week after collection of serum. B. Immunoelectron microscopy preparation of the serum in panel A. The
serum was mixed with matched convalescent-phase serum (final dilu-
tion convalescent-phase serum 1:100), incubated for 90 min at 37°C,
and virions/immune complexes centrifuged directly to a specimen grid
with the EM-90 rotor. Arrow, complete virion; arrowhead, genome-
defective virion; phosphotungstic acid. Bar = 100 nm. For study details,
see Plummer et al., 1985 (16).
troenteric agents may be necessary. These swab samples should be placed in capped conical centrifuge tubes with 0.2 mL sterile, distilled water, sealed with Parafilm, and sent for electron microscopic diagnosis. Lesion crusts should also be placed in sterile conical tubes. The addition of any liquid medium to lesion crusts, cerebrospinal fluid, nasopharyngeal washes, saliva, tears, and urine will not assist the electron microscope laboratory. Tissue biopsy samples in buffer without fixatives should be stored at 4°C and sent directly to both an electron microscope facility and a viral identification laboratory for rapid electron microscopy and other diagnostic testing. Fixation may interfere with antibody binding and thus preclude infectivity tests and successful application of any immunoelectron microscopy.

Finally, failure to collect an adequate volume of sample will limit the tests that may be used and the ability to successfully identify causative agents. Lesion fluids are deceiving. For example, samples containing poxvirus or varicella zoster virus that appear to have no material drawn into a needle barrel (Figure 3A) or attached to a grid may still contain numerous virions. When possible, at least 1 g of fecal material should be collected into a commercial stool collection vessel. A minimum of 5.0 mL of blood should be collected into tubes without anticoagulants. When a special interest in the case or outbreak occurs, large samples may provide reagents for later testing. All samples should be immediately sent for rapid electron microscopic diagnosis, with storage at 4°C if possible. Dried smears may be stored and transported at ambient temperature. Under no condition should samples be frozen for storage and transport before receipt at the diagnostic facility (30).

**Containment of Biological Hazards and Laboratory Safety**

Protecting staff and containing infectious agents are important considerations in the handling of all clinical specimens. Samples may contain agents that are highly infectious or associated with a high mortality rate. In consideration of the possibility of bioterrorism and agriterrorism, delivering samples to a central facility at biological safety level (BSL) 3 or higher may be necessary for inactivation before electron microscopic examination. Regardless, preparation must be done in a laminar flow hood with BSL-2 or greater containment capability. Most infectious agents may be inactivated in suspension by adding formaldehyde or glutaraldehyde (20 min, final concentration 2% and 0.5%, respectively). Alternatively, hazardous samples may be inactivated after they are mounted on the grid by treating the grid with fixative, by subjecting stained preparations to ultraviolet irradiation (UV) for 5 min before removing them from the biological safety cabinet, or both. UV treatment may, however, affect both virion morphology and grid stability. Prolonged treatment with glutaraldehyde or formaldehyde has little effect on morphology while inactivating most agents (31). Both formaldehyde and glutaraldehyde immobilize structures by Schiff reactions involving aldehyde side groups. As a di-aldehyde with a 5-carbon backbone, glutaraldehyde is more effective than formaldehyde at intermolecular crosslinking. Glutaraldehyde may, therefore, cause aggregation and obscure some fine structural detail. Samples suspected of containing spores should be inactivated with 10% formaldehyde final concentration because spores are more resistant to chemical inactivation (32). Specimens that may contain prions require more harsh treatment, such as the addition of 1 M NaOH, to inactivate the samples. However, treatment with NaOH will degrade most biologic structures to an indecipherable tangle of artefacts, and is, therefore, not conducive to electron microscopic examination.

Specimens that have not been inactivated must still be treated as potentially infectious after electron microscopic examination. For example, no decrease was observed in a 50% tissue culture infective dose (TCID<sub>50</sub>) of poliovirus samples after they were mounted on the grid and stained with 2.5 mM (1.6%) phosphotungstic acid, pH 7.0. Subsequent exposure to vacuum and the electron beam for 1 min reduced TCID<sub>50</sub> by at
least $10^{+5}$ and $10^{+4}$ for adenoviruses and polioviruses, respectively. More importantly, 10-min vacuum and electron beam exposure of grids containing sporulating B. subtilis preparations permitted colony recovery in 60% of tests and reduced colony-forming units 500-fold, and exposure to either vacuum or phosphotungstic acid-negative stain alone had little effect on the viability of adenovirus, poliovirus, or spore preparations. These observations underline the extreme resistance of spores in different weapons delivery systems. Because of the risk for residual infectivity, all grids must be disposed of as infectious waste, and equipment used to handle samples and grids, e.g., forceps, must be decontaminated by treatment with 5% glutaraldehyde for 20 min. Alternatively, equipment may be disinfected with 1 M NaOH. Cleaning is also necessary to prevent false-positive results caused by crossover contamination between specimens. Staff involved in rapid electron microscopy should be vaccinated for multiple agents, including smallpox and hepatitis B.

**Specimen Preparation**

While rapid electron microscopy may be performed with any type of specimen, the requirement for truly rapid electron microscopic diagnosis is not common. Indicators include limiting exposure in clinically threatening situations in which an infectious cause is not ruled out, as may occur if a patient has suspected herpetic lesions in a ward for immunocompromised, newborn, or transplant patients; new clinical symptoms are observed with immunocompromised patients; the need to initiate early treatment; or the risk of passing infection during birth. Since a viral agent may be found by rapid electron microscopy in over 90% of poxvirus and other skin lesions of viral etiology (Gelderblom and Hazelton, unpub. data), this method is ideal for investigating outbreaks of rash-like illness and suspected cases of bioterrorism.

A morphologic diagnosis may be obtained within 10 min of specimen arrival in the electron microscope facility. The standard two-step drop method, i.e., adsorption followed by negative staining, is used for preparation (Figure 4A). Viral load is usually more than sufficient to allow successful diagnosis of herpesvirus, poxvirus, and some gastroenteric infections. Negative-stain examination is simple and may be conducted in any electron microscope facility. The first item needed is a 400-mesh electron microscope grid coated with either a single plastic layer or a plastic film reinforced with carbon (32,35,36). Carbon-coated plastic films have higher thermal stability and are less prone to specimen movement during examination. However, they may be more hydrophobic than plain plastic films. Electron microscope units that specialize in virus preparative or diagnostic techniques prepare their own plastic-coated, carbon-stabilized films, and glow discharge the films to improve hydrophilicity, particle adherence, and distribution of both sample and stain (36,37). Coated grids may also be purchased through most electron microscopy suppliers. Clinical samples with high concentrations of protein often do not require glow discharge pretreatment to reduce hydrophobicity.

Lesion fluids received in the barrel of a needle or capillary tube are expelled onto a hydrophobic surface such as Parafilm. If the sample has dried, a small drop of redistilled water (15 µL), sterilized through a 0.2-µm-pore filter, is drawn into the specimen container and washed back out. If required, an aliquot of suspension should immediately be transferred to viral transport medium and submitted for cell culture, nucleic acid amplification techniques, and other virologic procedures. Lesion crusts and biopsy material may be soaked in 3 volumes of distilled water. Heavy debris is allowed to settle, and the suspension cleared by low-speed centrifugation (1,000 x g for 5 min). Liquid samples (cerebrospinal fluid, nasopharyngeal washes, saliva, tears, and urine) may be used directly. If required, aseptically transfer an equal volume of double concentration fixative may be mixed with the suspension to inactivate any infectious agents present before mounting the sample on the coated grid. A grid is floated with the coated surface on a drop of fixed suspension for 0.5-2 min and excess material wicked away with an edge of filter paper (Figure 4A,B). If bacteria are to be negatively stained, higher

![Figure 4](image-url)
numbers of microorganisms will attach to the grid because of sedimentation when the drop is placed on the grid. Adsorption is not an absolute process. Any extra manipulations, such as washing the grid, may reduce the number of adsorbed particles. Pretreating the carbon-reinforced grids by glow discharge, poly-L-lysine, alcian blue, or UV light may also help for tighter binding (32,35) and is particularly useful when staining aldehyde-inactivated samples. Direct touch lesion fluid preparations, which are already mounted on the grid, may be rehydrated and inactivated before staining by floating the grid on a drop of fresh 2% formaldehyde.

Rapid immunologic methods that improve sensitivity when searching for unknown agents include solid-phase immuno-electron microscopy (SPIEM) (38) and serum in agar (SIA) (39), both of which may use either pooled human immunoglobulins (HuIgG) or specific antibodies. HuIgG may be obtained from most immunologic suppliers or hospital pharmacies. SPIEM concentrates antigens on the grid by immune capture, thereby improving the probability of observing an etiologic agent. The coated surface of a grid is floated on a drop of pooled HuIgG (100 µg/mL and 20 µg/mL in phosphate-buffered saline [PBS] B) or antiserum (1/100 and 1/500 in PBS) for 10 min, washed on 6 sequential drops PBS, and floated on the specimen for 30–60 min at 37°C. The sample may be stabilized after SPIEM with 0.1% glutaraldehyde to ensure tight binding of the captured antigens, washed on 6 drops of PBS, negative stained, and examined (38). SIA uses immunoaggregation to identify antigens. In addition, type-specific antisera may be used in SIA to serotype the agent present. Antibody (1/100 for antisera and 100 µg/mL for HuIgG) is prepared in cooled 1% agar. A grid is placed on the solidified agar, and a drop of sample placed over the grid. Diluent diffuses into the agar while antibody diffuses into the suspension and antigen:antibody complexes form, which then adsorb to the grid as diluent volume is reduced (Figure 4B) (39).

**Negative Staining**

Biologic structures, because of low mass density, interact weakly with electrons used for imaging, and therefore, show little contrast or detail. Several ways exist to generate sufficient image contrast and resolution; the most versatile is positive and negative staining with heavy metal ions, e.g., lead, tungsten, and uranium ions. Positive staining depends on chemical reactivity with the components of the object and involves fixation, postfixation, embedding in resins, ultrathin sectioning, and multiple staining incubations. These procedures may take 4–5 days before a sample is ready for examination. Rapid embedding protocols can reduce the time to approximately 1 day but with a loss in specimen quality (32). In contrast, negative staining is simple, rapid, and well suited for examination of small particulate suspensions. A coated grid with sample adsorbed to the surface is floated on a drop of negative stain for 0.5–2 min, excess stain wicked away with a piece of filter paper, air dried for 1–3 min, and examined by electron microscopy (Figure 4D). Structures on the grid are surrounded and stabilized by the drying stain. Thus, they appear as transparent, highly detailed negative images within a dark halo of stain (Figure 5B).

The most common negative stains are 1% (60 mM) aqueous uranyl acetate, pH 2-4.5, and 1% (2.5 mM) phosphotungstic acid, pH adjusted to 7.0 with NaOH. Aqueous uranyl acetate is unstable at higher pH values. Because aqueous uranyl acetate and phosphotungstic acid differ in staining properties, both stains should be applied in parallel in case of unknown...
samples. Stains should be relatively fresh and stored in brown glass bottles at 4°C (32,35,36). While the stained grid is being examined, additional grids may be left floating on the sample droplet, protected from dust and drying. This method reduces preparation time in the event additional grids must be prepared for electron microscopic inspection.

Particle Enrichment

If no virus has been identified after 20 min or after the examination of 10 grid squares, the result may be considered to be “no etiologic agent identified.” Routine two-step drop preparations for electron microscopic diagnostic procedures require particle concentrations of $10^6$ to $10^8$/mL. Therefore, negative evidence is not an absolute diagnosis. A number of effective concentration or immunologic procedures exist that markedly increase sensitivity of electron microscopic diagnostics for samples with lower particle concentrations (32,40). These procedures take from 0.5 to 16 hours and are labor and training intensive. Viral research or diagnostic facilities generally have access to at least one advanced procedure. Nonimmunologic procedures include: a) ultracentrifugation concentration—the material from cleared suspensions is sedimented by ultracentrifugation, resuspended in a smaller volume and then prepared by the standard two-step drop method (32); b) agar diffusion—a 20–50 [20- to 50-µL drop of suspension is placed on 1% agar. As the fluid is absorbed the virus is concentrated. After 15–20 min, a grid is placed on the remaining suspension and then stained as with the two-step method above (Figure 4D). This procedure will result in an enrichment factor of approximately 5x (32); and c) direct centrifugation to the electron microscopic grid with the Beckman Airfuge (Beckman, Palo Alto, CA) EM-90 rotor or A-100 rotor, a procedure that increases sensitivity up to 1,000 fold (40–42). Immunoaggregation and immunodecoration with type- and genus-specific antibody may be used to concentrate material or to specifically identify the agent, e.g., herpes simplex 1 and 2 and varicella zoster. Also, convalescent-phase serum samples may be used to identify infectious agents or provide evidence of seroconversion to the agent when paired with acute-phase sera. For standard immunoelectron microscopy, the suspension is incubated for 1 h at 37°C with serum samples diluted in PBS, and then mounted on the grid by using either the drop method or direct centrifugation to the grid. Immunoaggregation may be very powerful in the identification of a suspected or novel agent or with small, dispersed virions (7,13,16). Immunoelectron microscopy was particularly useful in the initial identification of noncultivable agents such as hepatitis C, Norwalk virus, and Winnipeg virus (7,13,43). Detailed methods may be found in references (29,32,35,44).

As with all diagnostic laboratory procedures, diagnostic electron microscopy should be performed in a quality-controlled manner. For routine external quality control, the Konsiliarlaboratorium für EM-Erregerdiagnostik at the Robert Koch-Institut in Berlin has conducted an External Quality Assurance-EM Virus Program, which provides panels of specimens containing different agents, since 1994 (www.rki.de/INFEKT/CONSULLAB/EM-DIAG). More than 95 laboratories from 27 countries participated in EQA-EMV 11 during August and September 2001. Each laboratory used its preferred method for preparation (45). A review of results submitted from participating facilities indicated that 27 of 69 laboratories correctly identified all test samples, while an additional 28 successfully identified four of five positive specimens. A trend towards higher success existed among laboratories that used enrichment procedures (35 of 55) when compared with those that were less successful (4 of 14) (p=0.055). However, experience, as defined by years of service and number of samples examined annually, was another important success factor.

Identification of Viral Agents

Several major pitfalls exist in the identification of viral agents by negative stain electron microscopy. First, the failure to detect and identify an agent does not mean that it is not there. Second, if you look long enough and hard enough, you will eventually find something that resembles what you wish to find. Third, the presence of a single picture cannot validate the interpretation of morphology. While the diagnostician must not be afraid to find something novel, the finding must be real. One example is the observation of multiple particles with similar morphology. In addition, photographic records must be made for all possible positive identifications and reviewed to confirm the accuracy of the initial diagnosis. Further, when a particle is assigned to a proper virus family, reviewing the case may be necessary to identify the genus or strain. For example, not all samples with orthopoxvirus morphology will be smallpox (Figure 6). While natural infections of variola virus have been eradicated, many other orthopoxvirus continue to be found and identified, e.g., camel-, cow-, monkey-, mouse-, and vaccinia pox viruses (17,46). In addition, the molluscipoxvirus Molluscum contagiosum is morphologically indistinguishable from orthopoxviruses. Identification of Molluscum contagiosum was essentially non-existent in Winnipeg before 1983. With the growth of the immunocompromised sector of the population, the number of identifications increased to 6–10 cases per year until 1995, when the change in sampling methods from lesion aspirates to swab collection in transport medium resulted in a reduction to 1–2 Molluscum contagiosum identifications per year (Hazelton, unpub. data). Further differentiation of poxviruses into variola, vaccinia, cowpoxviruses, or molluscipoxvirus may be performed by immuno electron microscopy with type-specific antibodies. Appropriate antibodies and the latest nucleic acid amplification techniques are also available for this determination at the World Health Organization Collaborating Centers at the Centers for Disease Control and Prevention, and VECTOR, Koltsovo, Novosibirsk Region, Russia.

Future Impact of Diagnostic Electron Microscopy

Compared with other laboratory diagnostic methods, electron microscopy excels with respect to rapidity and the open
view that permits detection and identification of both novel agents and those not considered by the clinician. However, full exploitation of this potential requires early and coordinated application of electron microscopy with other frontline diagnostic procedures. The use of electron microscopy to examine diagnostic cultures of Hendra virus provided evidence of a paramyxovirus 3 days before any other results were available. Thus, focusing further characterization on the proper virus family was possible, and a novel pathogenic agent, which became the prototype strain for the henipah viruses, a proposed new genus of paramyxoviruses, was found (17,47). Diagnostic electron microscopy does not need to be either expensive or difficult to perform if executed in a diagnostic network, i.e., by recruiting instruments and electron microscopists working in other departments, e.g., cell biology or pathology (48). Respective arrangements are facilitated by using inactivated samples and implementing new technologies, such as automated pattern recognition (49) and telemicroscopy by using digital image acquisition and remote operation of the instrument or review of micrographs through the Internet (50).

As with smallpox diagnosis from the 1940s to the 1970s, electron microscopy differential diagnosis has often ruled out the occurrence of dangerous pathogens. The power to rapidly identify agents of bioterrorism has now been demonstrated convincingly by Tom Geisbert and Peter Jahrling, U.S. Army Medical Research Institute of Infectious Disease, when they identified and quantified spores in the B. anthracis bioterrorist letter attack upon U.S. Senate Majority Leader Daschle.

Figure 6. A–E. Comparison of clinically relevant viral agents associated with skin lesions. A–C show poxviruses indistinguishable in appearance from variola virus, the agent of smallpox. The slightly rounded, brick-shaped virions measure about 270 by 350 nm. Two types of particles may be seen. M, or mulberry forms show a 10- to 20-nm diameter short-tubular or beaded surface (M). Capsular, or C forms, partly penetrated by the stain, are recognized by a 30-nm membrane (C): A. Molluscum contagiosum (molluscipoxvirus) virions from skin lesions observed in an adult; B. Vaccinia virus vaccine strain WR (orthopoxvirus) from cell culture; C. Ectromelia virus (orthopoxvirus) from culture material. D. Parapox viruses measure up to 190 by 300 nm and are more distinctly ovoid. Tubules, 10 to 20 nm wide and approximately 1,000-nm long, spiral around the virion, giving a distinctive crosshatched appearance. E. Herpesvirus particles from a skin lesion of a primary varicella zoster infection observed in an adult. Direct electron microscopy shows two virions. The envelopes are broken, liberating the 100-nm nucleocapsid. F. Cell culture supernatant from a patient with an infantile respiratory tract infection. The enveloped virions are studded with tiny surface spikes. The 18-nm helical nucleocapsids have been released from disintegrating virions. The nucleocapsids and envelope details are typical of paramyxoviruses. A–B, phosphotungstic acid, C–F, uranyl acetate. All prints at the same magnification, bar = 100 nm.
SYNOPSIS

Figure 7. A colony of *Bacillus anthracis* was suspended, inactivated, and negatively contrasted with aqueous uranyl acetate, as described for Figure 4. The microorganisms, which grow in long chains, do not have flagella. B. The ubiquitous *B. subtilis* may also grow as long chains. However, in contrast to *B. anthracis*, the *B. subtilis* cells show distinct flagella (arrow). Bar = 2.5 µm.

(Jahrling, pers. commun.) (Figure 7). Because the unusual and unexpected can be rapidly identified, electron microscopy must remain a frontline method for rapid diagnostic virology, investigation of potential bioterrorist events, and investigation of new and unusual cases of suspected infectious origin.

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Dr. Hazelton is electron microscopist for the department of medical microbiology and infectious diseases, University of Manitoba, Winnipeg, Canada. He is also a virologist with over 20 years’ experience in electron microscopic viral diagnostics. His research interests include viral assembly and transmembrane transport, and the pathogenesis and epidemiology of gastroenteric viruses.

Dr. Gelderblom is head of the electron microscope and imaging group and the Konsiliarlaboratorium für EM-Erregerdiagnostik at the Robert Koch-Institut, Berlin, Germany. He is a virologist with over 30 years’ experience in electron microscopic viral diagnostics and an interest in structure-function studies of complex viruses. He and Dr. Hazelton have a mutual interest in rapid electron microscopic diagnostics and improved detection methods for electron microscopic diagnostics.

**References**


Appendix. Directory of electron microscope facilities which may provide assistance and advice concerning emergency viral diagnostic matters.¹

**Australia**
Alex Hyatt
Geelong, Victoria, Australia
Phone: 61 0352275419
Fax: 61 0352275555
e-mail: alex.hyatt@csiro.au

John Marshall
North Melbourne, Victoria, Australia
Phone: 61 3 93422678
Fax: 61 3 93422660
e-mail: john.marshall@mh.org.au

**Austria**
Wolfgang Muss
Salzburg, Austria
Phone: 43 662-4482-4720
Fax: 43 662-4482-882
e-mail: W.Muss@lks.at

Susanne Richter
Wien, Austria
Phone: 43 173216/5146, 47 173216/5107
Fax: 43 173216/5194
e-mail: Susanne.richter@bfl.gv.at

**Belgium**
Patrick Goubau
Brussels, Belgium
Phone: 32 02 7645492
Fax: 32 02 7645422
e-mail: goubau@mblg.ucl.ac.be; laboratoire.sida@mblg.ucl.ac.be

Marc van Ranst
Leuven, Belgium
Phone: 32 16 332160
Fax: 32 16 337340
e-mail: Marc.Vanranst@rega.kuleuven.ac.be

¹ Do not send samples to any facility without first contacting the facility for instructions; not all labs listed meet biological safety level 3 conditions; facilities may be required to refer queries to other, jurisdictional laboratories.
Brazil
Hermann Schatzmayr
Rio de Janeiro, Brazil
Phone: 55 21 598 4274
Fax: 55 21 270 6397
e-mail: Hermann@ioc.fiocruz.br

Marli Ueda
São Paulo, Brazil
Phone: 55 11-3068-2908
Fax: 55 11-3088-3753
e-mail: marliueda@hotmail.com

Canada
Hans-Wolfgang Ackermann
Laval, PQ, Canada
Phone: 1 418-656-2131, ext. 2558
Fax: 1 418-656-7666
e-mail: Ackermann@mcb.ulaval.ca

Paul Hazelton
Winnipeg, MB, Canada
Phone: 1 204-789-3313
Fax: 1 204-789-3926
e-mail: paul_hazelton@umanitoba.ca

Judith Isaac-Renton
Martin Petric
Vancouver, B.C. Canada
Phone: 1 604-660-6032
Fax: 1 604-660-6073
e-mail: judy.Isaac-renton@bccdc.ca

Raymond Tellier
Toronto, Ontario, Canada
Phone: 1 416-813-6592
Fax: 1 416-813-6257
e-mail: Raymond.tellier@sickkids.on.ca

Don Stoltz
Halifax, NS, Canada
Phone: 1 902-494-2590
Fax: 1 902-494-5125
e-mail: dstoltz@is.dal.ca
Czech Republic
Jana Schramlová
Prague, Czech Republic
Phone: 420 2 6708 2572
Fax: 420 2 7274 4354; 420 2 6708 2387
e-mail: jschraml@szu.cz

Denmark
Jens Blom
Copenhagen, Denmark
Phone: 45 3268 3578
Fax: 45 3268 3883
e-mail: jbl@ssi.dk

Finland
Olli Vapalahti
Helsinki, Finland
Phone: 358 9-19126604
Fax: 358 9-19126491
e-mail: olli.vapalahti@helsinki.fi

Carl-Henrik von Bonsdorff
Helsinki, Finland
Phone: 358 9-1912-6506
Fax: 358 9-1912-6491
e-mail: carl-henrik.vonbonsdorff@helsinki.fi

France
Pierre Gounon
Nice, France
Phone: 33 04 92 07 60 46
Fax: 33 04 92 07 60 45
e-mail: gounon@unice.fr

Germany
Stefan Becker, Larissa Kolesnikova, Hans Dieter Klenk
Marburg, Germany
Phone: 06421-2865433
Fax: 06421-2865482 or 06421-2868962
email: Stefan Becker becker@mailer.uni-marburg.de
       Larissa Kolesnikova kolesnick@mailer.uni-marburg.de
       Hans Dieter Klenk klenk@mailer.uni-marburg-de

Werner Eichhorn, Oskar-R. Kaaden
München, Germany
Phone: Eichhorn 49 89-2180 2531
Kaaden 49 89-2180 2535
Fax: 49 89-2180 2597
e-mail: werner.eichhorn@micro.vetmed.uni-muenchen.de

Bernhard Fleischer, Herbert Schmitz, Christel Schmetz
Hamburg, Germany
Phone: 49 40 42818 467, 49 40 42818 468
Fax: 49 40 42818400
e-mail: pcs@bni.uni-hamburg.de; bni@bni-hamburg.de

Hans Gelderblom, Reinhard Kurth, Georg Pauli, Andrea Männel
Berlin, Germany
Phone: H. Gelderblom 49 30-4547-2337
A. Männel 49 30-4547-2326
Fax: 49 30-4547-2334
e-mail: gelderblomh@rki.de

Harald Granzow, Th. C. Mettenleiter
Insel Riems, Germany
Phone: 49 38 351-7206
Fax: 49 38 351-7151
e-mail: Harald.Granzow@Rie.BFAV.de

Bärbel Hauröder
Koblenz, Germany
Phone: 49 261 896-7260
Fax: 49 261 896-7109
e-mail: b.hauroeder@zinstkob.de

K.-F. Reckling
Stendal, Germany
Phone: 49 3931-631818
Fax: 49 3931-631 153
e-mail: Reckling@lvluasdl.ml.lsa-net.de

India
Atanu Basu
Pune, India
Phone: 91 20-6127301
Fax: 91 20-6122669
e-mail: Atanu Basu atanu_b@hotmail.com
Milind Gore milind_gore@hotmail.com

Ireland
WW Hall, Patrick Costigan
Dublin, Ireland
Phone: 353 1-716-1338, 353 1 716-1354
Fax: 353 1-269-7611
e-mail: patrick.costigan@ucd.ie

**Israel**

Jossi Manor
Hashomer, Israel
e-mail: ymanor@sheba.health.gov.il

**Italy**

Guisy Cardeti, Nazareno Brizioli
Roma, Italy
Phone: 39 06 79099448
Fax: 39 06 97340724
e-mail: gcardeti@rm.izs.it

Carlo Chezzi
Parma, Italy
Phone: 39 0521-988885
Fax: 39 0521-993620
e-mail: clchezzi@ipruniv.cce.unipr.it

Massimo Gentile
Roma, Italy
Phone: 39 06 447441224
Fax: 39 06 447441236
e-mail: gentilemax@tiscalinet.it

Antonio Lavazza, Ezio Lodetti
Brescia, Italy
Phone: 39 30 229 0298
Fax: 39 30 242 5251
e-mail: alavazza@bs.izs.it

**Japan**

Toshiyuki Goto
Kyoto, Japan
Phone: 81 75 751-3925
Fax: 81 75 751-3909
e-mail: tgoto@itan.kyoto-u.ac.jp

Naomi Sakon
Osaka, Japan
Phone: 81 6 6972-1321
Fax: 81 6 6972-2393
e-mail: sakon@iph.pref.osaka.jp
Etsuko T. Utagawa  
Tokyo, Japan  
Phone: 81 3 5285-1111  
Fax: 81 3 5285-1161  
e-mail: etu@nih.go.jp

Netherlands  
Albert DME Osterhaus  
Rotterdam, Netherlands  
Phone: 31 10-4088066  
e-mail: Osterhaus@viro.fgg.eur.nl

Paul Roholl  
Bilthoven, Netherlands  
Phone: 31 30 2743651  
Fax: 31 30 2744437  
e-mail: p.roholl@rivm.nl

Russia  
Elena Ryabchikova  
Koltsovo, Novosibirsk Region, Russia  
Phone: 383 2-36-60-01  
Fax: 383 2-36-74-09  
e-mail: lenryab@vector.nsc.ru

Slovenia  
Mateja Poljsak  
Ljubljana, Slovenia  
Phone: 386 1 5437460  
Fax: 386 1 5437401  
e-mail: mateja.poljsak-prijatelj@mf.uni-lj.si

Spain  
Maria Inmaculada Herrera  
Majadohonda/Madrid, Spain  
Phone: 91 509 7969/01  
Fax: 91 509-7966  
e-mail: iherrera@isciii.es; alvarezh@teleline.es

Sri Lanka  
Mohamed Abdul Azeez Razak  
Colombo, Sri Lanka  
Phone: 94 1-693532, 1-693533, 1-693534, ext. 411  
Fax: 94 1-575405  
e-mail: azeezrazak@hotmail.com, medresit@slt.lk
Switzerland
Monika Engels, P. Wild, Elisabeth M. Schraner
Zurich, Switzerland
Phone: 41 1 635 8791
Fax: 41 1 635 8911
e-mail: emschra@vetanat.unizh.ch

Thomas Baechi
Zürich, Switzerland
Phone: 41 1 634 26 65
Fax: 41 1 634 49 06
e-mail: baechi@emz.unizh.ch

United Kingdom
A. Barry Dowsett
Porton Down, UK
Phone: 44 0 1980 612247
Fax: 44 0 1980 611096
e-mail: barry.dowsett@camr.org.uk

Bill Cooley
Addlestone, United Kingdom
Phone: 44 0 1932 357824
Fax: 44 0 1932 357659
e-mail: w.a.cooley@vla.defra.gsi.gov.uk

United States
John Bozzola
Carbondale, IL, USA
Phone: 1 618-453-3730
Fax: 1 618-453-2665
e-mail: bozzola@siu.edu

Joan Dragavon
Seattle, WA, USA
Phone: 1 206-341-5210
Fax: 1 206-341-5237
e-mail: dragavon@u.washington.edu

Carol E. Hearne, Donal O’Toole
Laramie, WY, USA
Phone: 307-742-6638
Fax: 307-721-2051
e-mail: CEHearne@uwyo.edu
       Donal O’Toole@wyo.edu
Charles Humphrey, Cynthia S. Goldsmith
Atlanta, GA, USA
Phone: Charles Humphrey 1 404-639-3307
        Cynthia Goldsmith 1 404-639-3306
Fax: 1 404-639-3043, 404-639-1377
e-mail: Charles Humphrey: cdh1@cdc.gov
        Cynthia Goldsmith: csg1@cdc.gov

Peter Jahrling
Frederick, MD, USA
Phone: 1 301-619-2772
Fax: 1 301-619-4625
e-mail: peter.jahrling@det.amedd.army.mil

Sara E. Miller
Durham, NC, USA
Phone: 1 919-684-3452
Fax: 1 919-684-3265
e-mail: saram@duke.edu

Frederick A. Murphy
Davis, CA, USA
Phone: 1 530-754-6175
Fax: 1 530-752-2801
e-mail: famurphy@ucdavis.edu

Robert W. Nordhausen
Davis, CA, USA
Phone: 1 530-752-8760
Fax: 1 530-752-6253
e-mail: rwnordhausen@ucdavis.edu