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Electron microscopy in rapid viral diagnosis: an update

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SUMMARY

Diagnostic electron microscopy (DEM) has conceptual predecessors – the application of the sense of vision and of light microscopy in medicine. The evolvement of DEM and the role of its two branches - histopathology and rapid negative-contrast DEM - are described in this review, with a focus on the latter. By its resolving power of 2 nm in praxi, DEM is able to visualize all kinds of pathogens, bacteria, parasites, even the smallest viruses. In contrast to other laboratory diagnostic methods, DEM excels by speed and "open view". All structures on the support grid can be assigned directly by "pattern recognition" of their fine structure to a specific family of agents. The morphology-based "catch-all" diagnosis can be decisive as a differential diagnosis and will help as a preliminary diagnosis to select and apply proper diagnostic tools for typing of the observed agent. Based on two case reports, the advantages and possible pitfalls of DEM are exemplified and hints are given to make DEM reliable and effective. Finally the role of DEM in medicine and the wider fields of life sciences are described together with the organizational conditions to guarantee its future in laboratory diagnostics.

KEY WORDS: Diagnostic electron microscopy, Negative staining, Rapid visualization of infectious agents, Pattern recognition, Morphology-based diagnosis, External quality assurance.

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INTRODUCTION

Automated diagnostic plants and nucleic acid-based detection (NAD) assays have taken over a wide sector in medicine and veterinary medicine. Agent-specific antigens, antibodies or parts of the agent's genome are detected as indicative of the specific infection: molecular techniques excel by high throughput, the ease of standardization and documentation and a hitherto unknown high sensitivity - advantages that helped to drive out diagnostic electron microscopy (DEM) from a number of its previous domains in clinical histopathology and virology. In the 1980s, DEM was widely used in particular in virology, pathology and medical laboratory services but today, the high equipment costs for DEM and the need to maintain an experienced staff are hampering its timely renewal. When senior scientists in DEM retire, the respective laboratories are often closed down when there is no trained in-house successor available (de Haro and Furness, 2013). Moreover some decision-makers tend to cultivate the opinion of DEM being a subjective and demanding, i.e. a "fossil" method that would be better replaced by objective, automatable techniques. Considering the sound achievements in molecular diagnostics and today's cost restraints, we feel the need to discuss anew the role and potential of DEM in microbiology, in virology in particular. We will do this on the background of the historic development and describing two paradigmatic diagnostic cases we will outline a few essential properties of DEM.

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There is no doubt, that DEM appears to be in retreat. Visualization, however, the principle of DEM, is unique by speed, "open view" and certainty. We will point out that DEM is an indispensable tool in infectious disease emergencies and wherever there is a need for rapid high resolution visualization, e.g., in process control of biopreparations,

PRINCIPLES AND ORIGIN OF DEM

Despite much progress in automated imaging techniques, "the naked eye", the sense of vision, is still widely used in medicine, e.g., as a *prima vista diagnosis* taking into account the general appearance of a patient and searching for pathognomonic skin lesions in a case of a febrile rush disease. From the ancient times of the Etruscans, Greeks and Romans, visual perception was used to judge the quality of urine and feces in order to recognize the underlying disease (Figure 1). The "naked eye" inspection became less important when chemistry and light microscopy were introduced - together they allowed diagnosis and differentiation, e.g., renal infections in an objective way by detecting both protein and bacterial contamination in the urine. Light microscopy raised great expectations as Robert Hooke (1635-1703) stated in his preface to his Micrographia (1665): "by the help of the microscopes, there is nothing so small, as to escape our inquiry" and Antoni van Leeuwenhoek (1632-1723), using single lense microscopes attaining up to 270-fold magnification, described bacteria for the first time (for a review see c.f.: Krüger *et al.,* 2000).

FIGURE 1 - *"The physician" ("Der Arzt", 1653) by Gerrit Dou (1613-1675) with permission of Kunsthistorisches Museum, Vienna. Until the late Middle Ages all five senses were used in medical assessment and attendance with the sense of vision dominating also in laboratory diagnostics, here in uroscopy.*

In the second half of the 19th century, mankind was still threatened not only by poverty, hunger and war, but also by deadly diseases. Diphtheria, tuberculosis, smallpox, plague and cholera caused endemic infections and/or major outbreaks with high mortality. The cause of what we know today as infectious diseases was uncertain, e.g., tuberculosis was considered a hereditary or constitutional entity, while other diseases were considered non-particulate, volatile miasmata. But, progress in science and technology were ripe to mount new approaches to elucidate the cause of contagious diseases, - an essential step before one could think about rational therapies.

Visualization and the Koch postulates

In his pioneering studies on the cause of anthrax ("Die Ätiologie der Milzbrandkrankheit", 1876) Robert Koch (1843-1910) combined three methods for the first time:

1) microscopy to describe the constant presence of a morphologically defined germ in infected tissues and in blood;
2) isolation of that germ using artificial cultures;
3) the reproduction of the disease in an experimentally infected animal.

Using artificial slide cultures, Koch also ob-

served the growth of anthrax bacilli, the formation of long filaments and of spores and documented his observations in detailed hand drawings (Figure 2). Feeling the need for a more precise and objective documentation and knowing about the potential of photography from his own experience, he soon adopted photomicroscopy (Koch, 1877). He used advanced equipment and methods, e.g., Abbe's condenser illumination and homogeneous oil immersion lenses allowing the highest numerical apertures and useful magnifications up to 1.000-fold as soon as they became available from the Carl Zeiss company (Figure 3). By his studies on tuberculosis in 1882, within a period of only eight months, Koch provided evidence that:

1) in every affected organ the same bacillus was present as judged by light microscopy;
2) the same type of bacillus was constantly isolated in pure culture;
3) after inoculation into guinea pigs the isolate induced tuberculosis (Koch, 1882).

The fulfilment of the three conditions, later defined as the three postulates (Loeffler, 1884) - also termed Koch-Henle postulates as discussed by Brock (1988), - proved that tubercu-

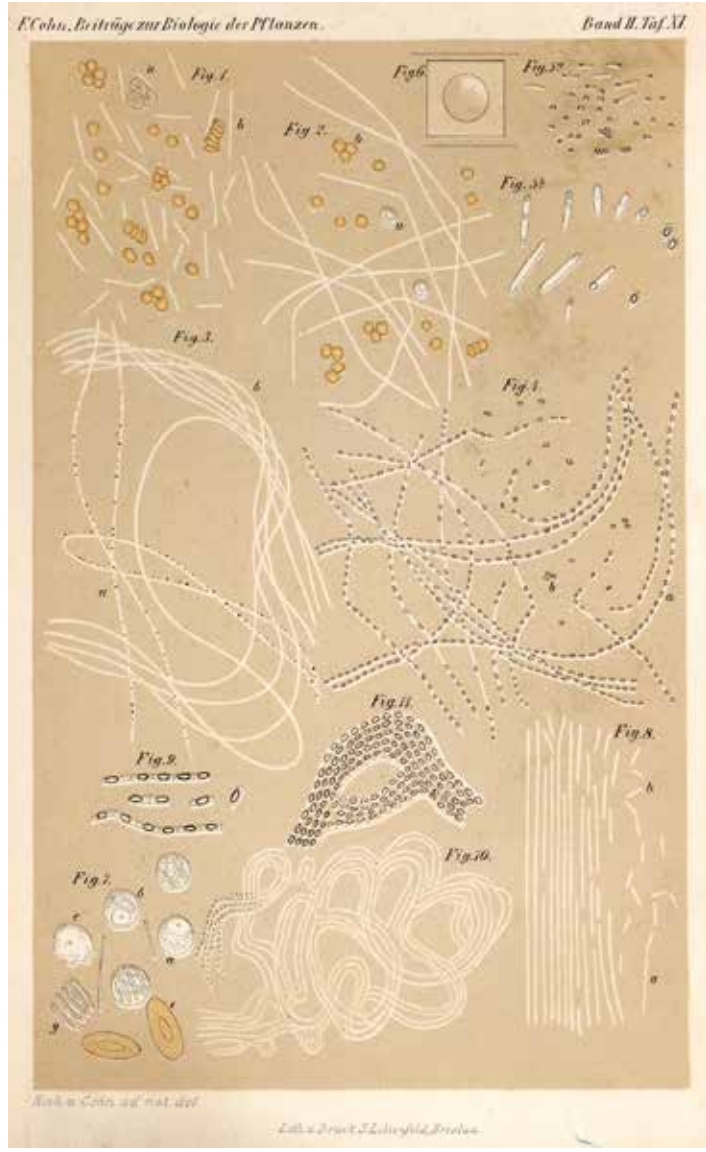

FIGURE 2 - *The table shows the development of Bacillus anthracis (Figs. 1-7; "Entwicklungsgeschichte von Milzbrandbazillen", Koch, 1876; magnification = 650X) and consists of Koch's hand drawings of his observations over the course of time. Figs. 8-11 represent different development stages of Bacillus subtilis, a mobile bacillus as depicted by Ferdinand Cohn. The bacilli appear as translucent, glass-like rods - occasionally kinked; (a) white blood cells (b) red blood cells. Figure 1B. - Anthracis visualized directly from the blood of an infected guinea pig. Figure 2 - A mouse spleen isolate of B. anthracis 3 hours after inoculation in aqueous humor of a rabbit's eye. Later Koch used the eye fluids of cattle for artificial cultivation of B. anthracis. The rods of bacilli are growing to partly kinked or bended filaments. Figure 3 - Same as Figure 2, after 10 hours' incubation. Bacilli have grown to long filaments part of them running in intertwined bundles. (a) Individual filaments contain refractive granula - to become spores later - at defined distances. Figure 4 - Same as Figure 2 after 12 hours' incubation: inside the filaments ovoid spores are seen at regular intervals; (b) some broken filaments and released spores showing a tendency for clumping. Figure 5 - Germination of spores: (5a, Koch) individual spores are growing to a cylindrical bacillus, with the refractive parts of the spore still placed on one pole. The spore material soon appears to shrink and disappear completely. (5b) Same specimen, but drawn by Ferdinand Cohn, the influential mentor of Robert Koch - using his higher resolving microscope. Figure 6 - A culture of B. anthracis cultivated in a heated slide culture stage in humor aqueous. This way Koch tested the influence of temperature and of aerobic and anaerobic conditions on bacterial growth and sporulation. In the upper right quadrant of the circular culture Koch observed the growth of delicate, whitish masses of filaments. Figure 7 - Experimental infection of a frog inoculated subcutaneously with a homogenate of a B. anthracis-infected mouse spleen. (a-e) Different cells with intracellular bacteria as well as free bacteria (g) not related to B. anthracis. Figures 8-10 - Different stages of Bacillus subtilis documented by Ferdinand Cohn using his higher resolving light microscope (magnification = 1650X).*

losis was indeed caused by the difficult to stain bacillus and served as the blueprint for the detection of further bacterial pathogens (for a review see c.f.: Brock, 1988). The achievements of bacteriology created great optimism that the burden of life-threatening infections could become eradicated in the near future. However, the era of bacteriology ended already in 1898 when Loeffler and Frosch described the cause of foot-and-mouth-disease (FMD) as an infectious, though invisible agent. Highly diluted samples of FMD vesicle fluids turned out to be infectious in consecutive animal transfer experiments, pointing to an infectious cause and ruling out the possibility of a soluble venom or toxin. Infectivity, however, was not held back by bacterial-tight filters, not sedimented by routine centrifugation and remained undetected even by advanced light microscopy: i.e. the FMD agent was much smaller than bacteria. Loeffler and Frosch postulated that other medical conditions like smallpox, cowpox, typhus, Rinderpest etc., might also be caused by similar minute organisms (Loeffler and Frosch, 1898). As FMD was conceptually similar to tobacco mosaic virus (TMV; Beijerinck, 1898; Ivanovski, 1892; Mayer, 1886) these and other hitherto negatively defined germs were summarized under the term virus.

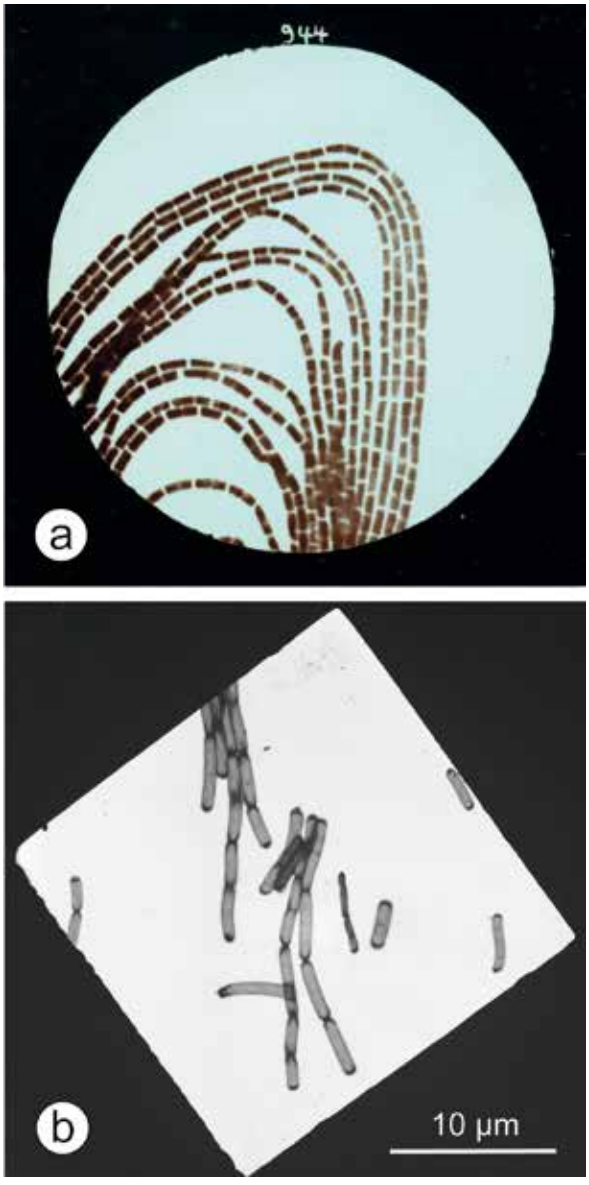

FIGURE 3 - *(a) Photomicroscopy of B. anthracis by Robert Koch showing bacilli forming long, parallel running chains. (b) DEM of B. anthracis. Negative staining with 0.5 % uranyl-acetate (UAc) of a suspension prepared from a colony. Most of the cells are still in continuity, no fimbriae detectable (negative-nr.: EM-10 # 67705). The grid bars of the copper support grid are blocking the electrons and form an open 30 x 30 µm in square which is covered by a thin translucent Pioloform film and reinforced by carbon to bear the specimen.*

DEVELOPMENT OF ELECTRON MICROSCOPY (EM)

To visualize viruses an entirely new type of microscope was required. Early in the 1930s accelerated electrons were shown to have a 10,000-fold shorter wavelength than that of visible light, and they could be focussed using electro-static or - magnetic lenses. In 1931, Ernst Ruska (1906-1988), the 1986 Nobel prize winner in Physics, built the first two-step magnifying transmission EM and recorded the first electron micrographs, i.e. a 16-fold enlarged metal grid (Knoll and Ruska E., 1932). Already in 1932, 12,000 - fold magnification was attained using lenses of a very short focal length that had been developed in close collaboration with Bodo von Borries (1905-1965) thus clearly surpassing the resolution attainable in light microscopy (Ruska E., 1934; for review see Haguenau *et al.,* 2003; Ruska E., 1980).
The further development of the new instrument was delayed for several years due to the

economic crisis after World War I, but finally due to a decisive intervention from medicine, it was continued. Helmut Ruska (1908-1973), the younger brother of Ernst Ruska and trained as a physician, motivated his clinical teacher Richard Siebeck to compile a professional assessment of the novel instrument. By October 2, 1936, Siebeck predicted "a direct practical significance for the physician":

1) Deeper insights into the fine structure of the body would allow a better understanding of normal and pathological structure and function.
2) The etiology of virus diseases, from smallpox, chickenpox, measles, mumps, influenza, Egyptian eye disease to foot-and-mouth disease could be clarified.
3) Also tumor virus research and phage studies would advance by the help of the electron microscope (Siebeck: in Ruska E., 1980).

Based on Siebeck's assessment and a favourable patent situation the Siemens & Halske company in Berlin under the direction of von Borries and Ernst Ruska resumed the development of the new instrument and started its commercial production in 1937 (regarding the efforts of other groups see c.f.: Freundlich, 1963; Gelderblom and Krüger, 2014; Haguenau *et al.,* 2003; Hawkes, 1985).

By 1938 the first electron microscopic studies on bacteria and viruses were published: size and fine structure of smallpox, vaccinia virus and Ectromelia mouse pox (von Borries *et al.,* 1938a, b), plant viruses like tobacco mosaic virus (Kausche *et al.,* 1939), and phages (Ruska H., 1940) were characterized and bacteria, their inner constituents and fimbriae visualized. At the Siemens "Laboratorium für Übermikroskopie" besides all kinds of microbes connective tissues and blood cells were also studied - it served also as a guest laboratory for many scientists from Europe (reviewed in: Gelderblom and Krüger, 2014).

ELECTRON MICROSCOPY AND VIRUS CLASSIFICATION

In the 1940s, the nature of viruses, their ways of propagation and pathogenicity had remained grossly unclear and hence virus classification followed conservative rules. Viruses were classified over decades phenomenologically, i.e. according to their hosts and the clinical symptoms they caused. For example, the "hepatitis" viruses comprised various agents belonging to different families - as we know today - different in morphology, strategies of infection, and pathogenicity (for detailed information see c.f.: ICTV, 2011). In 1943, Helmut Ruska described the agents of chickenpox and zoster, i.e. two human herpesviruses, as morphologically identical, spherical particles 140-150 nm in diameter, often with a central body and structurally clearly different from the much larger, brick-shaped pox viruses (Ruska H., 1943a). Aggregating all structural information available, he proposed a novel, science-based virus classification (Ruska H., 1943b). Taking the size and shape of the agents as the decisive criteria, he established virus families, different from each other, but morphologically uniform in themselves. Probably due to World War II, this pioneering approach was not seen outside Germany. A comparable virus classification – using then, however, virus morphology and nature of the viral nucleic acid - was established not earlier than 1962 (Almeida, 1963; Lwoff *et al.,* 1962).

MILESTONES IN DIAGNOSTIC ELECTRON MICROSCOPY (DEM)

The potential of EM in the laboratory diagnosis of infectious diseases was shown first in the control of smallpox outbreaks (Nagler and Rake, 1948; Rake *et al.,* 1948; van Rooyen and Scott, 1948). Its reliability and speed remain valid today (Goldsmith and Miller, 2009; Goldsmith *et al.,* 2013; Long *et al.,* 1970; Madeley, 2003; Miller, 2003; Hazelton and Gelderblom, 2003). By December 9, 1979, WHO certified "that smallpox has been eradicated from the world" following global vaccination campaigns (for review see: Fenner *et al.,* 1988). WHO took great care that variola virus stocks kept so far in many virus laboratories were destroyed or transferred into one of two high security WHO controlled Reference Centers: at the CDC, Atlanta, USA, and at VECTOR in Koltsovo, Russia, respectively, but basically remnants from smallpox biowarfare programmes as well as

other high risk agents like Yersina pestis and Francisella tularensis (Lane *et al.,* 2001; Morens *et al.,* 2004), or live virus in an innocent, "forgotten" deep freezer, even genetically engineered infectious molecular constructs could be used for a bio- or agro-terrorist attack (Brumfield, 2003; Gewin, 2003; Lane *et al.,* 2001; LeDuc and Jahrling, 2001). The probability of a bioweapon attack (Lane *et al.,* 2001; Morens *et al.,* 2004) appears low, but cannot be ruled out completely. Therefore public health offices need to maintain a high level of preparedness to counter bioterrorism, including measures for a rapid diagnosis of the agents. When applied properly, DEM turned out to be reliable (Long *et al.,* 1970; Peters *et al.,* 1962; Reed *et al.,* 2003). Also when in the two-year smallpox post-eradication period in 1980-81 more than 4000 dubious febrile vesicular rashes were analyzed, DEM exclusively revealed members of the herpesvirus family, thus ruling out smallpox (Fenner *et al.,* 1988).

Also more recently, DEM proved decidedly successful, e.g., in the search for the SARS agent at the CDC (Ksiazek *et al.,* 2003), the diagnosis of monkeypox virus infections in the USA (Reed *et al.,* 2004) and of Hendra paramyxovirus infections in Australia (Murray *et al.,* 1995; Hyatt *et al.,* 2001). In 2001, it was also routinely applied in the laboratory diagnosis of anthrax after the intentional release of anthrax spores following the 9/11 terrorist attacks in the USA (personal communication Peter Jahrling, USAMRIID, 2002; Jernigan *et al.,* 2002).

The role of EM in the evolving field of virology

EM and virology are furthering each other from their beginning in the 1930s to date (Krueger *et al*. 2000). Viruses, relatively simple organized agents, were the challenge to develop the high-resolution instrument while virology benefitted by the description of new agents, insight into the virus-host relation and control of diagnostics and classification. By the early 1960s a new generation of easy to use EMs and reliable negative staining (NS) techniques were introduced (Brenner and Horne, 1959; for technical information see c.f.: Biel and Gelderblom, 1999a; Bozzola and Russel, 1999; DeCarlo and Harris, 2011; Field, 1982; Flewett, 1985; Hayat and Miller, 1980; Harris, 1997; Madeley and Field, 1988; Maunsbach and Afzelius, 1999). "New" agents, e.g., adeno-, entero-, myxo-, paramyxo-, and reoviruses were isolated in diagnostic cultures (Almeida, 1963; Tyrell and Almeida, 1967; for a review see c.f.: Goldsmith *et al.,* 2013) and grouped as morphologically distinct agents. "Fastidious", i.e. not cultivable agents were detected when "dirty" specimens like plasma, urine and feces were studied (Almeida, 1983; for a review see c.f.: Madeley, 1995): the viruses of hepatitis B and hepatitis A (Dane *et al.,* 1970; Feinstone *et al.,* 1973); rotaviruses causing epidemic gastroenteritis (Bishop *et al.,* 1973; Flewett *et al.,* 1973); astro-, calici- and noroviruses linked with outbreaks of diarrhea and vomiting (Kapikian *et al.,* 1972; Madeley, 1979). EM became a favoured tool also in the fields of plant virology and phage classification (Ackermann and Prangishvily, 2012; Wright, 2005; ICTV, 2011). EM was applied to differentiate retroviruses (Bernhard, 1960; Gelderblom *et al.,* 1974) and later to define HIV by its unique morphology as a lentivirus - the Lentivirinae forming a genus of the retrovirus family (Gelderblom and Pauli, 1986; Gelderblom *et al.,* 1987; Gonda *et al.,* 1985). Currently more than 30,000 different viruses have been described. Based on genetic properties and on morphology they are classified into 93 virus families or orders. Specific for vertebrates are 28 families, 22 of them are found with humans ICTV, 2011).

As the genotype of an agent determines its phenotype, its morphology, the visualization of morphological criteria of bacteria, viruses, or parasites directly implies the identification of a suspect particle as a member of a particular family of agents - or as a member of a hitherto new virus family, as experienced recently with the mimivirus (La Scola *et al.,* 2003). Virus families differ from each other in many aspects, the simplest being size: from 17 nm for porcine circovirus (Tischer *et al.,* 1982) to 150 x 200 x 300 nm for the brick-shaped poxviruses (Fenner *et al.,* 1988) to 400 nm for mimiviruses (La Scola *et al.,* 2003). Due to the immediate diagnosis, DEM excels by speed. Including a routine negative stain preparation, a diagnosis can be attained within 15 min after the arrival of the sample in the DEM laboratory. As the sense of

vision (Figure 1) appears the most important among the five human senses, DEM is based on a robust mental sensorium.
DEM provides an instant diagnosis as to the family type of the agent - in contrast to many test alternatives that diagnose a virus down to the type and species level. However, the family-based DEM diagnosis can help the clinician as a differential diagnosis to initiate treatment and/or isolation measures without the need for further, time-consuming virus typing. But also when the type of the agent needs to be known, e.g., for forensic reasons, DEM helps to select appropriate agent-specific molecular assays. Finally, DEM typing can also be achieved by immunoelectron microscopy (Feinstone *et al.*, 1973; Field, 1982; Goldsmith and Miller, 2009; Kurth and Nitsche, 2007; Miller and Howell, 1997, Wright, 2005).

Sample collection and preparation
Biosafety regulations require careful inactivation management during sample collection and preparation and routine skills in handling infectious and/ or otherwise hazardous substances (for details see: CDC, 2014; Hayat and Miller, 1990; Madeley and Biel, 2006; Gelderblom *et al.*, 2007). Samples may be collected from a wide spectrum of sources: directly from the patient as vesicle fluids, crusts or biopsies, all kinds of body excretions, diagnostic cell cultures (Goldsmith and Miller, 2009; Goldsmith *et al.*, 2004, 20011, 2013), or environmental samples (Gelderblom, 2003; Hazelton and Gelderblom, 2003; Kurth *et al.*, 2008). Before taking routine biopsies to examine suspicious skin exanthems, one should resort ahead to the less harmful NS-DEM (Gelderblom *et al.*, 2006), while biopsies are preferentially diagnosed by time-consuming thin section histopathology. With warts and similar conditions it will be worthwhile to apply NS after grinding the tissue and making a suspension, preferentially in distilled water. Cotton swabs are less well suited for the collection of EM diagnostic samples as their preparation involves dilution steps (Gelderblom and Hazelton, 2000).
Preparation should be conducted under BSL 2 or higher safety conditions, i.e. in a Class 2 Laminar Flow Hood. Although the amount of material sticking to a grid represents less than 0.1 microL of the diagnostic suspensions, "out-diagnosed" grids also need to be inactivated. Details have been described (CDC 2014; Gentile and Gelderblom, 2005; Hazelton and Gelderblom, 2003).

Principles and technical aspects of DEM
Conventional transmission EM (TEM) and routine NS preparation can attain *in praxi* a spatial resolution of 2 nm. Thus all structures bigger in size than 2 nm are visualized, including the smallest viruses or other nanoparticles. To present the fine structure of an agent, merely a simple and robust contrast staining is required. For this goal heavy metal salt solutions are used - there are no agent specific reagents involved. Therefore, the "open view" of DEM can visualize all agents on the specimen grid, also unknown microbes as well as unsuspected ones. DEM is a "catch all method", different from other methods in the diagnostic repertoire (Almeida, 1983; Biel and Gelderblom, 1999b; Field, 1982; Flewett, 1985; Gelderblom *et al.*, 1991; Goldsmith and Miller, 2009; Hazelton and Gelderblom, 2003; Madeley, 1995, 2003; Madeley and Field, 1988; Miller, 1986; Roingeard, 2008; Zhang *et al.*, 2013).
The preparation in DEM follows basically one of two simple routines: NS-TEM or the preparation of ultrathin sections (TS-TEM). Rarely shadow-cast preparations are used. NS, used to prepare suspensions of particles, is the fastest method in virology. In contrast, TS-TEM uses resins to dehydrate and infiltrate small blocks of organs, tissues or cells after double-fixation and staining. After polymerization of the resin, ultrathin sections 40 to 80 nm in thickness are prepared and post-stained using electron-dense heavy metal salt solutions. In TS-TEM the different chemical groups of the specimen react to various degrees with the heavy metal salts – based on chemical affinities. Due to the complex preparation scheme, this histopathology type of DEM requires *in praxi* near 10 days (de Haro and Furness, 2012; Schroeder *et al.*, 2006), though recently also shorter TS-TEM schemes were established (see later). NS-TEM, in contrast, allows a sound diagnosis within a few minutes as there are no chemical affinities involved, at least not in the usual short-term preparation scheme.

Negative staining of virus suspensions

Introduced in the early 1960s (Brenner and Horne, 1959), the procedure consists basically of three steps: adsorption of particles to a support film, washing and staining - as described in much detail in textbooks and papers (Bozzola and Russel, 1999; De Carlo and Harris, 2011; Biel and Gelderblom, 1999 a; Gelderblom *et al.,* 1991; Gelderblom, 2003; Gentile and Gelderblom, 2005; Goldsmith and Miller, 2009; Hayat and Miller, 1990; Harris, 1997; Hazelton and Gelderblom, 2003; Maunsbach and Afzelius, 1999). Rather then discussing minute technical details, we would like to mention merely two essentials: successful NS-TEM requires hydrophilic grids and a concentration of $>10^5$ particles per ml in the diagnostic suspension. The latter condition is often met in clinical samples, e.g., vesicular rashes caused by herpes - and poxviruses - but also many other agents exceed this lower limit by several orders of magnitude. Viruses like other biologicals consist mainly of light atoms that do not induce an appreciable contrast in the conventional TEM. To visualize biologicals with high contrast, heavy metal salt solutions are used. At least 4 to 5 "stains" can be recommended (for details see c.f.: Biel and Gelderblom, 1999a; De Carlo and Harris, 2011; Harris, 1997; Hayat and Miller, 1990). The metal salt solution surrounds all structures on the grid and after drying down encases them as a very thin, glass-like, electron-dense "envelope". The stain may also penetrate labile structures - particularly true for phosphotungstic acid (PTA), while uranyl acetate (UAc) tends to stabilize labile, especially enveloped viruses. PTA and UAc differ widely in staining properties. Therefore at least these two should be used: if required in parallel, especially when dealing with unknown samples. After a few minutes of preparation and air-drying, the grid is ready for examination in the EM, i.e. a diagnosis can be made within 15 to 20 min.

Only small amounts of a diagnostic suspension are required. A minimal volume of 1 or 2 microL will suffice, however, larger volumes are preferred as they will allow for particle enrichment techniques or immuno-EM. In case of low particle titers, enrichment methods bring down the threshold to 10^4 and 10^3 particles per ml, e.g., sedimentation by the Airfuge^R directly onto the grid (Biel and Gelderblom, 1999a; Gelderblom and Reupke, 1978; Hammond *et al.,* 1981) or still more sensitive combinations of immune-aggregation and sedimentation (Biel *et al*., 2004; Gelmetti *et al.,* 1996).

Two steps are essential in NS-DEM: diagnostic suspensions require a low speed "clear centrifugation" before being adsorbed to the grid: 1.000-2.000g for 10 min will remove broken cells and/or bacteria that otherwise may hide the structures of interest. Secondly, the quality of the support grids is most critical. Mechanically and thermally stable support films are required - made of Pioloform^R or Formvar^R and reinforced with a thin layer of carbon. The carbon surface must be adhesive, otherwise particles from the suspension do not adhere tightly and are washed off during NS. The required "stickiness" of the carbon is achieved reliably by a glow discharge pre-treatment (Aebi and Pollard, 1987). Other means for a proper surface ionization are pre-treatment with poly-L-lysine or Alcian-Blue (Biel and Gelderblom, 1999a; De Carlo and Harris, 2011; Harris, 1997; Hayat and Miller, 1990). To control proper quality, the grids should be prepared and ionized in the DEM laboratory itself. Interestingly, grids used in DEM can be analyzed later also by nucleic acid amplification techniques (Johnsen *et al,* 2006) - a way to type the observed virus rapidly, but also to control DEM internally.

Figure 4 demonstrates a clinical case and the respective DEM. On April 17, 1998, on a Friday afternoon one of us (HG) was called by the clinician Professor Hans D. Pohle to assist in diagnosis and therapy. The patient, estimated 25 to 35 years in age, had been admitted to the Hospital in a febrile state without consciousness and lesions all over the body – most of them with clear content and in the same state of development (Figure 4a). without any papers for identification. He showed vesicular Smallpox appeared to be ruled out: "eradicated from the world" already in 1979 (Fenner *et al.,* 1988). But also animal orthopoxviruses, like cowpox- , mouse- and monkeypox virus can induce severe, even life-threatening zoonoses - in particular in immuno-compromised patients (Czerny *et al.,* 1991; Reed *et al.,* 2004). From the tenderness of the thin-walled vesicles, however, a generalized case of chickenpox, zoster or herpes simplex

appeared more likely. Before giving an antiviral the advice of physicians ruled out the possibility of a generalized allergy. Two vesicles - one on the face and one on the trunk - were opened and two EM grids per vesicle touched down into the fluid right to the bottom of the lesion. After air-drying the grids were taken across the street to the DEM laboratory. NS-TEM revealed an abundance of particles typical of the herpesvirus family (Figure 4b) in both lesions: damaged envelopes and isometric capsids around 100 nm in diameter. A poxvirus infection and an allergic state therefore appeared highly unlikely and the instant therapy with initially three grams of Aciclovir^{R} was started. The life-threatening brain swelling and fever receded within six hours and the patient recovered completely due to the still timely gift of the drug. Antivirals are effective, but not trivial drugs because of inherent long-term side effects. Therefore the indication to give an antiviral should be based on robust evidence. DEM was achieved within 15 min and given the life-threatening state of the patient the role of DEM in this emergency cannot be underestimated.

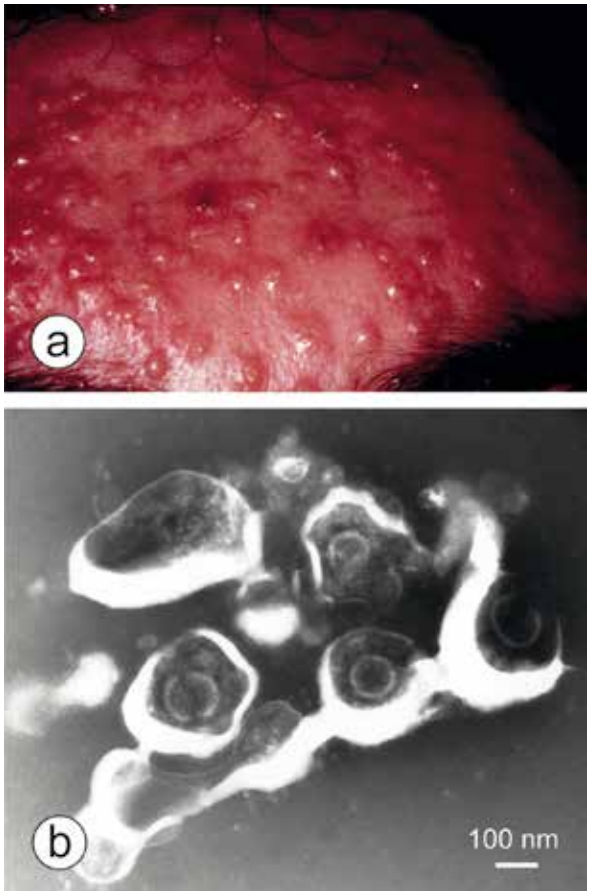

FIGURE 4 - *(a) The forehead of a febrile patient admitted to hospital without consciousness bearing numerous vesicles. They appear relatively thin-walled with the majority of them at the same development state and containing clear fluid. Two vesicles were opened for DEM and two electron microscopic support grids touched down into the vesicle fluid. (b) DEM after negative-staining with 1% sodium phosphotungstate (PTA) revealed an abundance of particles of the herpesvirus family, the majority of them with broken envelopes and released virus nucleocapsids. The "deep embedding" of virus particles in the electron dense "stain" caused severe particle damage and shrinkage and breakage of the stain. These drying artefacts, however, do not interfere with a safe diagnosis (negative-nr.: EM-10 # 57359).*

DEM, however, does not rely only on proper sample preparation, but also on experience and diagnostic expertise as shown in Figure 5 and described in full detail elsewhere (Nitsche *et al.*, 2007). The paper originates in a collaboration between two Public Health Laboratories. In February 2006, colleagues in Austria had studied hemorrhagic skin lesions on the hand of a farmer. He suffered from a "pronounced reddish rash" with "deep red to purple confluent plaques". NS-DEM revealed "bacteria in a clean background and seven profiles, 260 nm x 400 nm in size" (Figure 6a), but no other virus-like particles. As the "profiles" resembled the outline of orthopoxviruses (OPV) it was decided to contact - within the consulting DEM framework - the Robert Koch Institute in Berlin for further clarification. The next day, three different samples were sent: "5 microL of hemorrhagic vesicle fluid, barely visible pieces of skin from the vesicle roof, and a scraping from the lesion base dried down on a paper sponge". The same day, DEM and PCR were performed in Berlin with three positive PCR and two positive DEM results. The particles observed, however, were ovoid, 260 nm in length and 150 nm in width, "completely surrounded by bundles of parallel surface threats" as known of members of the parapoxvirus genus (PPV) (Figure 6b). The homogenized vesicle skin showed 10^{7-8} PPV particles/ml, the eluate from the sponge only 10^{4} particles/ml, while the vesicle fluid sample - disappointingly - failed to show any virus. Remarkably, the PPV positive DEM grids showed decent amounts of detritus

(Figure 6b), while vesicle fluid-grids appeared "clean" and "empty".

PCR proved unequivocally the presence of PPV with $1.7x10^3$ genome equivalents (ge) in the vesicle fluid, $2.3x10^6$ ge for the vesicle roof, and $3.1x10^2$ ge for the sponge eluate. Thus PCR and DEM ruled out the initial DEM-based suspicion of an OPV infection and arrived instead at the correct diagnosis of PPV. Considering the time required for preparation and evaluation, DEM was faster than PCR. Summarizing the performance of both detection techniques it was proposed that "in infectious disease emergencies both DEM and PCR should be applied front-

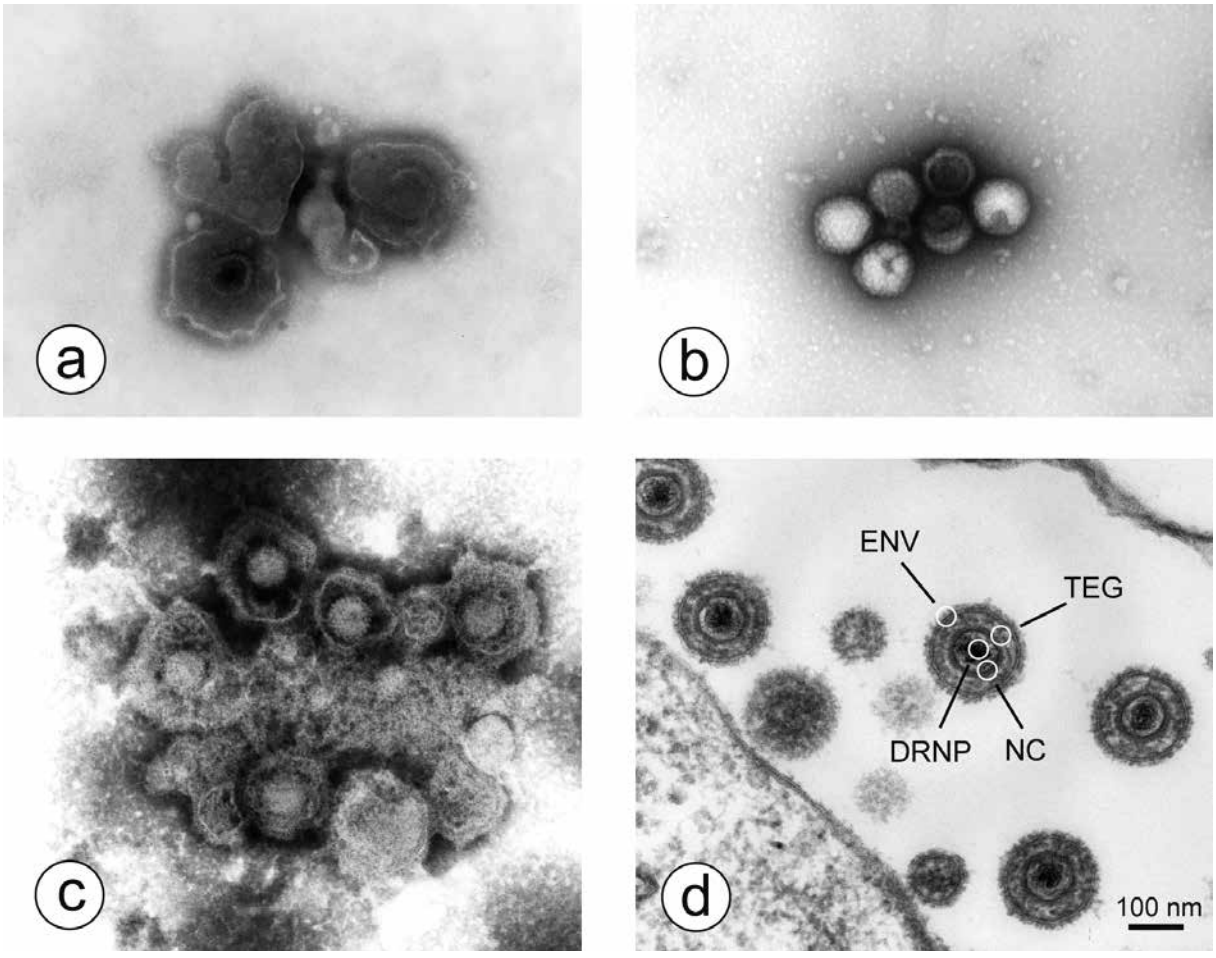

FIGURE 5 - *Comparative aspects of herpesviruses: (a) Vesicle fluid prepared directly from the patient shown in Figure 4a. As the PTA-layer is much thinner than in Figure 4b, the virus particles are relatively well-preserved. The envelopes are flattened and the cores or nucleocapsids inside the virions barely detectable (negative-nr.: EM-10 # 57360). (b) DEM showing six "naked" virus capsids after UAc "staining", three of them revealing evidence of a capsomers substructure while the other capsids are penetrated by the stain (negative-nr.: EM-10 # 73565). (c) Immuno-typing of a human herpesvirus isolate using using a polyclonal envelope specific anti-HSV-1 antiserum diluted 1:20 (Biel and Gelderblom, 1999a). After 30 min incubation at 37°C, the mixture was directly contrasted with 1% PTA. The viral envelopes appear densely coated with antibodies and 6 or more virus particles are cross-linked forming a diagnostically significant immuno-aggregate (negative-nr.: S-101 # 27397). (d) Thin section electron microscopy, based on a section thickness of typically 40 to 70 nm thickness, often reveals more insights into complex structures. Inside the herpesvirus virion, the DNA-genome is protected as a very-electron dense desoxyribonucleoprotein (DRNP), which itself is guarded inside the isometric nucleo-capsid (NC). The NC is coated by the tegument layer (TEG) followed by the viral envelope (ENV (negative-nr.: EM-10 # 52208).*

line – in parallel - to lower the risk of making a misdiagnosis due to faulty sampling, preparation and interpretation" (Nitsche *et al.,* 2007).

What can we learn from the initial "pitfall" in DEM?

How come the initial misinterpretation of OPV?

Two questions need to be discussed here:

1) what were the "OPV-like structures" observed initially;
2) why did they lead directly to the misinterpretation of OPV?

There is no doubt the profiles observed (Figure 6 a), are reminiscent of OPV. However, their

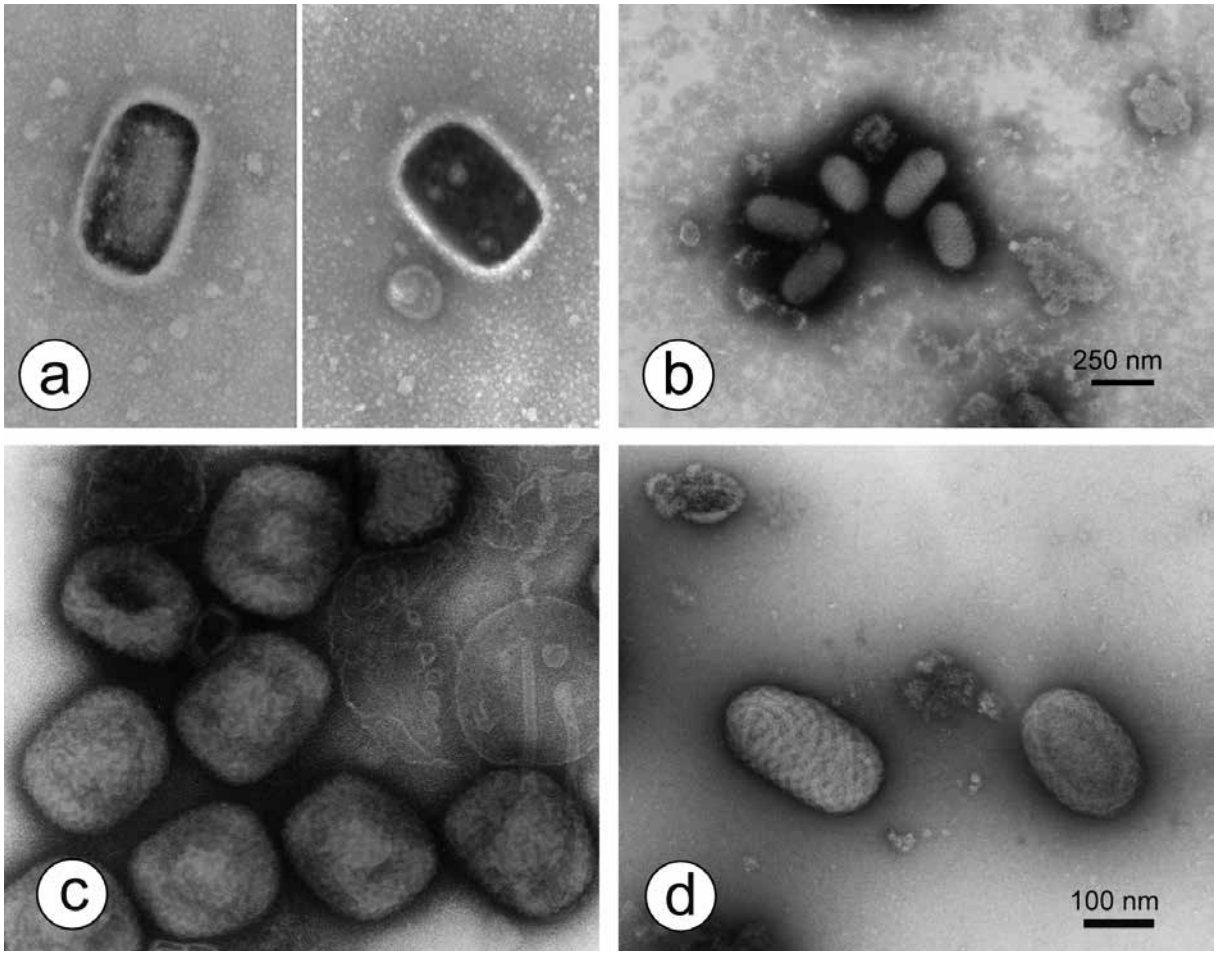

FIGURE 6 - *From the paper: "Pitfalls in DEM" (Nitsche et al., 2007) to illustrate and discuss possible mistakes in preparation and assessment. (a) Two elongated "profiles" measuring 260 x 400 nm with rounded corners are seen on a relatively clean "empty" support grid. As the profiles are reminiscent of the contour-lines of orthopoxviruses (OPV) they raised suspicion and alarm. (b) Diagnostic samples were send to the Robert Koch Institute for further evaluation and revealed a high concentration of parapoxvirus (PPV) particles accompanied by an appreciable amount of detritus. Vesicle roof after thorough grinding, Airfuge sedimentation onto the grid, PTA-staining (negative-nr.: EM-10 # 76546). (Figs. 6a and 6b reprinted with kind permission of ELSEVIER). Based on pattern recognition, members of the OPV and PPV subfamilies were immediately differentiated by negative stain DEM. (c) OPV particles, e.g., camelpox, cowpox, mousepox, smallpox, vaccinia, monkeypox viruses appear "brick"-shaped. PTA-staining. Measuring 250-400 x 150-250 nm, they are considerably larger than the elongated PPV with 250-300 x 150 nm (d). Besides size and shape also the surfaces of OPV and PPV differ clearly. While OPV (c) show short surface ridges, PPV particles, e.g., Orf, bovine papular stomatitis and pseudocowpox virus reveal surface spirals surrounding in parallel the complete virion - UAc staining. (c) negative-nr.: EM-10 # 68659; (d) negative-nr.: EM-10 # 64293)*

faint appearance and the absence of a defined surface structure should have cast doubts on their viral nature. Moreover, the "clean" background indicated that only little - if any - material stuck to the grid. A "clean background" in DEM must raise doubts as to the specimen and/or to the preparation ("stickiness of the grid"). Except for highly purified specimens, all DEM specimens - be they clear vesicle fluid, culture supernatants, or biopsy and stool suspensions - contain detritus: small molecular dirt (Figure 6 b) as well as larger, particulate contaminations that reflect the biological origin of the sample. Therefore, grids in DEM should reveal some detritus. The "lack of dirt" together with the low-contrast appearance of the "OPV profiles" disprove the initial "OPV" diagnosis. The profiles are simply holes in the plastic film of the specimen grid - partly stain-filled. Holes may often arise when films are prepared under too humid conditions. They are, as also here, often similar in size and shape and represent a preventable artifact.

But why did DEM in Berlin fail to detect PPV in the vesicle fluid - in contrast to PCR? A likely explanation: the sample had been diluted 500-fold in buffer in the General Laboratory before being handed over to the DEM group. The current $1.7x10^3$ genome equivalents, i.e. too high a dilution, prevented the third successful PPV-positive DEM diagnosis, an assumption consistent with the fact that the grids did not show any detritus.

Recommendations for proper sample collection and preparation

Details are found in many textbooks and papers (Biel and Gelderblom, 1999a; Bozzola and Russel, 1999; De Carlo and Harris, 2011; Field, 1982; Gelderblom and Hazelton, 2000; Gentile and Gelderblom, 2005; Goldsmith and Miller, 2013; Hayat and Miller, 1990; Hazelton and Gelderblom, 2003; Maunsbach and Afzelius, 1999; Stirling *et al.*, 2012). Atlases on the morphology of viruses and bacteria contain useful, though limited and in part also outdated information (Horne, 1974; Madeley and Field, 1988; Palmer and Martin, 1982). Therefore, the particulars of DEM are preferentially learned during a stay in an expert laboratory. In addition, we would like to draw attention to merely a few specific points that we found helpful in our own DEM work.

1) Specimens must be accompanied by a data form sheet with clinical information relevant to both the patient and the diagnostic specimen. Data are required as to the person: name and affiliation, age and sex, the quality of symptoms and their onset, date of sample collection, the origin/site and quality of the sample, the address including telephone and e-mail of the sender to facilitate rapid data exchange.
2) In a bedside situation the "direct touch method", i.e. the sampling of vesicle fluids directly on the grid is most effective. In case of pox- or herpesvirus infections, the lesion base will contain a maximum of virus load. Vesicle fluids and/or other suspensions can be transported to the DEM laboratory in capillary glass tubes, dried down on microscopic glass slides or in small plastic vials (Gelderblom, 2003; Gentile and Gelderblom, 2005). Infectious material should be packaged in a shatterproof container, accompanied by the data sheet.
3) Samples for DEM and the other laboratory diagnostic methods should be collected in parallel. If applicable: more than one specimen per patient in order to study different lesions. A carefully considered and documented sampling will support a safe and rapid diagnosis. If possible, the DEM staff should be involved in sample collection.
4) DEM is basically simple in theory and methods. Vertebrate viruses are clustered in merely 28 genetically and morphologically defined virus families/classes with clear-cut differences in morphology. As one of us (HG) experienced when operating an External Quality Assurance Scheme in EM Virus Diagnostics (EQA-EMV), DEM expertise can be acquired without much difficulty provided the newcomer is already experienced in general life science TEM. Given this experience and a positive motivation, the initial study of a few reference papers, a one week collaboration in an expert laboratory, regular participation in an EQA program, attendance in respective Workshops and Lab-Courses and a decent sample throughput will guarantee a robust competence in DEM.

5) A "clean" grid in DEM - without any "dirt" - must raise suspicion as it points to a lack of hydrophilicity ("stickiness") of its carbon layer. Hydrophobic surfaces will not bind biologicals. Except for highly purified samples, all diagnostic suspensions contain some bio-debris. The "dirt" on the grid proves proper grid quality as well as adequate procedures. The required hydrophilicity can be checked directly by observing the spreading behaviour on the grid's surface of a droplet of water or buffer.
6) Positive controls may be used, especially after an unexpected negative DEM result or when only low particle titers are likely in the sample. A defined volume of a marker molecule admixed to the sample can verify the performance of the preparation. To determine dimensions and concentrations, "internal" markers, e.g., catalase crystals, colloidal gold, latex particles of known sizes and concentrations, even purified adenovirus particles are used (Gentile *et al.,* 1994; Luftig, 1968; Zheng *et al.,* 1996).
7) Routine DEM relies primarily on NS techniques. In certain situations, however, TS-TEM should also be applied, e.g., when information on the virus-cell-interaction is required or when particularly labile agents are studied (Goldsmith and Miller, 2009; Hazelton and Gelderblom, 2003; Miller, 2003). Conventional TS-TEM may need up to 10 days (de Haro and Furness, 2013) while recent rapid embedding techniques allow for a same day-diagnosis. Rapid embedding can be performed with little extra effort by laboratories executing already routine TS-TEM (Laue *et al.,* 2007; Schroeder *et al*., 2006; Zechmann *et al*., 2011).
8) Immuno-EM (IEM) combines the high resolution of TEM with the marvellous biodiversity of the immune system. Monoclonal or polyclonal agent-specific antibodies may be utilized in DEM in a number of more or less complex IEM techniques. Humble immuno-aggregation using a pool of unlabelled hyperimmune-sera will facilitate the detection of even unknown agents. Provided avidity and titer of the antibodies suffice, IEM is reliable as the following references may show (Biel and Gelderblom, 1999a; Bertiaume *et al.,* 1981; Feinstone *et al.,* 1973; Field, 1982; Gelderblom *et al.,* 1985, 1987; Gelderblom and Schwarz, 1976; Goldsmith and Miller, 2009; Kapikian *et al.,* 1972; Miller and Howell, 1997). IEM helps in virus enrichment on the grid (Biel and Gelderblom, 1999a; Gelmetti *et al.,* 1996), in typing and in the high-resolution localization of antigenic sites (Beutin *et al.,* 2005; Schwarz *et al.,* 1983).
9) In DEM a grid should be screened for 20 min (or 10 fields on the 400 mesh grid) in order to detect low titer agents as well as multiple infections. As DEM cannot be performed in an automated way, its success depends widely on experience and dedication. We know of a DEM laboratory shouldering in the past an exceptionally high diagnostic burden of near 10,000 samples per year. Nevertheless, DEM is not well-suited for screening such a large numbers of samples. As alternative molecular methods have taken over much of the routine diagnostics, DEM now rightly focuses on the more urgent cases.

Is there a need for additional, sophisticated preparation procedures in DEM?

In conventional NS the adsorbed particles are flattened to various degrees and also the fine structure of labile agents may suffer because of the final rigid air-drying step involved. One therefore could argue about the use of cryo-techniques to improve structure-preservation. Indeed, they do prevent drying artifacts (Adrian *et al.,* 1998; De Carlo *et al.,* 2002; De Carlo and Harris, 2011; Harris, 1997). However, they require a cryo-transfer system and a TEM run under cryo-conditions - prerequisites that are neither sufficiently robust nor foolproof in a DEM routine.

Moreover, cryo-TEM is exceedingly time-consuming and indeed, time and throughput are limiting factors in DEM.

On the contrary, NS-DEM does benefit from particle damage! Herpes-, paramyxo- and other complex viruses will present diagnostically significant internal structures - capsids and capsomeres, the "herring-bone" ribonucleoprotein, respectively - only when the viral envelopes are damaged and permeable to the negative stain (Figures 5 and 7).

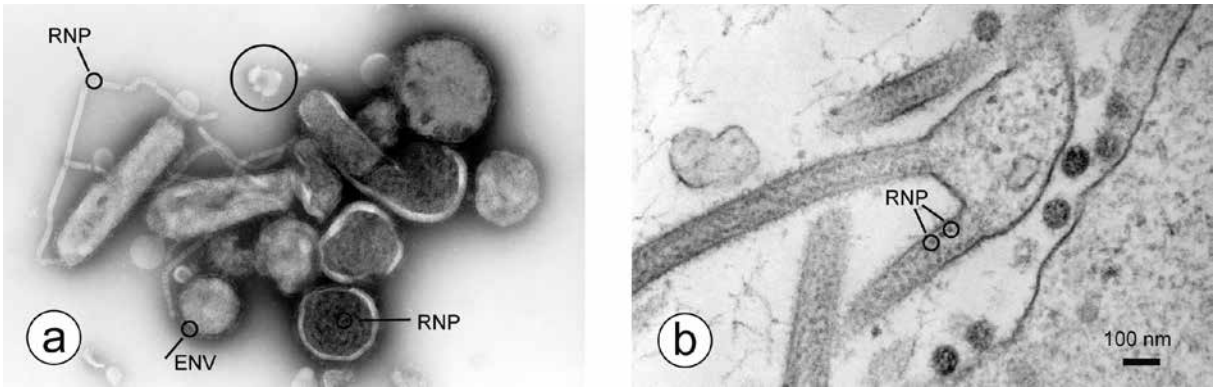

FIGURE 7 - *Virus particles produced in a VERO E6 cell culture 24 hours after inoculation of a SARS coronavirus isolate. (a) Culture supernatant was inactivated by 4% of paraformaldehyde and after Airfuge sedimentation onto the support grid stained with 1% PTA. The sample revealed abundant masses of paramyxovirus (PMV) particles. PMV can be highly pleomorphic – either spherical up to 400 nm in diameter or more filamentous in shape. PMV particles bear short envelope projections – detectable here at their periphery. Inside stain-penetrated virions, strands of the viral ribonucleoprotein (RNP) can be detected. When the labile viral envelopes are broken, RNPm strands, 18 nm in diameter with a herring-bone pitch structure are released. Encircled: a single coronavirus virion, recognizable by its irregularly spaced, but prominent peplomers and its diameter of 90 nm (negative-nr.: EM-10 # 71917). (b) Part of a VERO E6 cell from the culture analyzed in Figure 7a. Ultrathin section TEM after rapid embedding (Schroeder et al., 2006). Four PMV filaments are seen budding from the cell surface. They appear rigid and their envelopes are studded with barely visible virus envelope projections. Inside the filaments cross-sections of the viral RNP can be recognized, more or less clearly depending on the section angle. In addition, at least five SARS-coronavirus particles are seen adhering to the cell surface: in thin section TEM they are characterized by spherical shape, a size of 90 nm, electron-dense material directly underneath the viral envelope and only faint envelope projections. While the virologists were interested in growing pure, high titer progeny of SARS-coronavirus, control by NS- and TS-TEM revealed contamination of the VERO cells by a PMV: a contamination common also in number of other permanent cell lines, e.g., HeLa, Hep 2, LLC-MK2. (negative-nr.: EM-10 # 75615).*

What about the role of other accessory techniques in DEM? X-ray spectroscopy can help to identify spores in DEM (Laue and Fulda, 2013) and electron tomography revealed significant internal virus structures that had remained hidden in conventional TEM (Mast and Demeestre, 2009). No doubt, this gain in insight can support a safe diagnosis when, e.g., a suspected particle is surrounded by too much electron-dense stain.

In tomography a particle is analyzed from all directions and even in proper slices. However, as accessory techniques depend on additional, expensive and time-consuming instrumentation, it might be more rewarding to search for further suspects on the grid revealing their morphology more convincingly.

As pointed out by Rossmann (2013), Subramaniam *et al.* (2007) and recently by Henderson *et al.* (2012) electron tomography, in particular cryo-tomography is a leading edge technique in structural research enabling the generation of 3D images at near-atomic resolution. DEM, however, does not need this ambitious resolution! Structure-preserving cryo-TEM does not help in rapid DEM as it interferes with the need for diagnostic speed.

The costs of DEM

To establish DEM de novo is expensive. An average TEM will cost between 250,00 and 600,000 €. An ultramicrotome requires an investment of 30,000 to 40,000 €, an automated embedding apparatus about 19,000 €. To operate two TEMS in parallel in a DEM unit appears advantageous because of maintenance and capacity issues. Thus, it appears nearly impossible to fund de novo a DEM laboratory that is able to refinance this investment. Furthermore, DEM also requires expertise and skills and a staff that appears difficult to bring together in a de novo situation. To in-

vest in already existing EM facilities will prove more rewarding. They should be reinforced and supported, e.g., by replacing outdated instrumentation. In DEM often older electron microscopes are operated, often >25 years old, run with conventional film cameras, and only poorly - if at all - supported by industry regarding maintenance and spare parts. It appears more realistic to invest in new, fully digitalized instruments and in motivated, younger personnel that can be trained in handling the instrument, in sample preparation and in diagnostic skills appears (Biel and Madeley, 2001; DeHaro and Furness, 2012; Tiekotter and Ackermann, 2012). Aside from the costly equipment and the need for a dedicated staff, the costs of running DEM are remarkably low: the preparation of an NS sample amounts to less than 0.5 € which is much less than the costs for alternative molecular tests. Accordingly, a not too small throughput of DEM samples is also cost-effective, as the overhead technical costs per sample will be reduced.

How to organize DEM?

In contrast to the majority of the other laboratory diagnostic methods, DEM depends very much on human factors, expertise and motivation. A daily flood of stool samples and/or screening day for day all fractions of a number of density gradients will be a weary job for the microscopist. When samples for DEM are not properly selected, the number of false "negatives" will increase and too many negative samples will cause frustration. Motivation will fade and DEM inevitably will become less effective. Attention and motivation are kept high by "interesting" samples, when DEM is run closely linked with the Department of Laboratory Diagnostics and/or when it develops its own,independent links to e.g., a Clinic of Infectious Diseases. The latter will facilitate access to patients and samples that are collected in an adequate way. And no doubt, when the sector enjoys priority, DEM can be performed in parallel to its own strong biomedical structural research program (Biel and Madeley, 2001). A regular throughput of diagnostic samples, >5 to 10 per week, will guarantee the quality of DEM by the experience generated and regular participation in EQA and educational measures will help to acquire and ensure it (Gelderblom, 2001, 2003).

The future of DEM

DEM is rapid. It does not depend on complex and time-consuming methods and whenever applied expertly, DEM succeeded in the diagnosis of a variety of infections (Hazelton and Gelderblom, 2003; Ksiazek *et al.,* 2003; Long *et al.,* 1970; Reed *et al.,* 2003). All kinds of samples can be analysed by EM, either directly from the patient (Hazelton and Gelderblom, 2003; Madeley, 2003; from diagnostic cell cultures (Goldsmith and Miller, 2009; Goldsmith *et al.,* 2004, 2013), or as environmental samples (Gelderblom, 2003; Hazelton and Gelderblom, 2003; Kurth *et al.,* 2008). The performance of DEM is improved further by digitalization (Tiekotter and Ackermann, 2012) and remote control microscopy (Schroeder *et al.,* 2001). It can benefit greatly from rapid embedding techniques (Schroeder *et al.,* 2006; Laue *et al.,* 2007; Zechmann *et al.,* 2011).

Due to its unique diagnostic approach - visualization - DEM appears useful. The molecular techniques that have taken over most routine viral diagnosis, may fail due to inhibitory substances (Kurth *et al.,* 2008), wrong primer use or to a change in the antigenic make-up. And often with "rare" agents, in particular in veterinary medicine, no alternative commercial tests are available. Therefore, the "open view" of DEM should be available in infectious disease emergencies, also with immuno-compromised patients, in suspects of agro- and bioterrorism as well as wherever questions can be answered by high resolution imaging. DEM must be used front-line in order to benefit from an early visualization and in parallel to the molecular tests (Curry, 2003; Curry *et al.,* 2006; Goldsmith and Miller, 2009; Miller, 2003; Hazelton and Gelderblom, 2003). When properly performed, DEM will excel by inherent speed and reliability (Biel and Gelderblom, 1999b; Biel and Madeley, 2001; DeHaro and Furness, 2012; Curry, 2003; Curry *et al.,* 2006; Gelderblom, 2003; Gentile and Gelderblom, 2005; Goldsmith and Miller, 2009; Goldsmith *et al.,* 2013; Hazelton and Gelderblom, 2003; Madeley, 2003; Miller, 2003; Peters *et al.,* 1962; Roingeard, 2008). Besides national centralized

DEM units, local facilities should also be run to guarantee a rapid and close networking. Considering the prevailing economic shortages, it would appear justified to combine DEM for medicine and veterinary medicine in joint teams.

Ties to academia will make DEM more lively and successful. By supporting university-based structural research, students may enter student-, diploma- and thesis-work in DEM and thus might become able and motivated successors in DEM. When results obtained in DEM and in structural research are published in due course - if possible in a number of ways - both academia and DEM will benefit (Biel and Madeley, 2001). Care should be taken that only high quality images are presented at proper sizes, a policy that scientific journals too often are no longer following (Ackermann, 2014). Besides the obvious value of a rapid DEM for the patient's or animal's health, the public will also learn about the wide spectrum of tasks that EM can fulfil in academia, industry and other institutions. A multi-task application of EM appears prudent - the costs of the expensive equipment and the dedicated staff are balanced this way by a rewarding output, by public esteem and better chances for funding and grant application. "Embedding" DEM in a fine structure research group in public health institutions, academia or industry will help for proper funding and assure a safe and sound future.

Essential information on DEM

Information on Training Courses, Meetings, on the External Quality Assurance Schemes in EM Virus Diagnostics and advice for handling of DEM samples, including suspected bioterrorism samples, can be obtained from Dr. Michael Laue, Robert Koch Institute, Berlin (E-mail: Lauem@rki.de). Addresses of expert DEM laboratories may be obtained here, too. Information on NS preparation can also be downloaded from the websites of the Centers for Disease Control and Prevention (www.bt.cdc.gov/labissues/index.asp and click on "Packaging Protocols for biological Agents/Diseases" and "Negative Staining Electron Microscopic Protocol for Rash Illness"; www.bt.cdc.gov/agent/smallpox/lab-testing/ and click on "Specimen Collection Guidelines".

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REFERENCES

Aebi U., Pollard TH.D. (1987). A glow discharge unit to render electron microscopic grids and other surfaces hydrophilic. *J. Electr. Microsc. Techn.* **7**, 29-33.

Ackermann H.-W., Prangishvili D. (2012). Procaryte viruses studied by electron microscopy. *Arch. Virol.* **157**, 1843-1849.

Ackermann H.-W. (2014). Sad state of phage electron microscopy. Please shoot the messenger. *Microorg.* **2**, 1-10.

Adrian M., Dubochet J., Fuller S.D., Harris J.R. (1998). Cryo-negative staining. *Micron.* **29**, 145-160.

Almeida J.D. (1963). A classification of virus particles based on morphology. *Can. Med. Assoc. J.* **89**, 787-798.

Almeida J.D. (1963). A classification of virus particles based on morphology. *Can. Med. Assoc. J.* **89**, 787-798.

Almeida J.D. (1983). Uses and abuses of diagnostic electron microscopy. *Curr. Top. Microbiol. Immunol.* **104**, 147-157.

Beijerinck M.W. (1898). Over en contagium vivum fluidum als oorszaak van de vlesiekte der tabaksbladen. *Vers. Gew. Verg. Wiss en Natuurk. Afd., Kon. Aka. Wetensch. Amsterdam.* **7**, 229-235.

Bernhard W. (1960). The detection and study of tumor viruses with the electron microscope. *Canc. Res.* **20**, 712-727.

Berthiaume L., Alaain R., McLaughlin B., Payment P., Trépanier P. (1981). Rapid detection of human viruses in faeces by a simple and routine immune electron microscopy technique. *J. Gen. Virol.* **55**, 223-227.

Beutin L., Strauch E., Zimmermann S., Kaulfuss S., Schaudinn C., Männel, A., Gelderblom H.R. (2005). Genetic and functional investigations of fliC genes encoding flagellar serotype H4 in wild-type strains of Escherichia coli and in a laboratory E. coli K-12 strain expressing flagellar antigen type 48. *Bio.Med. Cent. Microbiol.* **5**, 4

Biel S.S., Gelderblom H.R. (1999a). Electron microscopy of viruses. in: Virus cell culture - a practical approach, Ed. A.J. Cann, Oxford University Press. 111-147.

Biel S.S, Gelderblom H.R. (1999 b). Diagnostic electron microscopy is still a timely and rewarding method. *J. Clin. Virol.* **13**, 105-119.

Biel S.S., Madeley D. (2001). Diagnostic virology - the need for electron microscopy: a discussion paper. *J. Clin. Virol.* **22**, 1-9.

Biel S.S., Nitsche A., Kurth A., Siegert W., Özel M., Gelderblom H.R. (2004). Detection of human polyomaviruses in urine from bone marrow transplant patients: a comparison of electron microscopy with PCR. *Clin. Chem.* **50**, 306-312.

Bishop R.F., Davidson G.P., Holmes I.H., Ruck B.J. (1973). Virus particles in epithelial cells of duodenal mucosa from children with acute nonbacterial gastroenteritis. *Lancet.* **2**, 1281-1283.

Bozzola J.J., Russel L.D. (1999). Electron Microscopy: *Principles and Techniques for Biologists*. Second edition, Jones and Bartlett, Boston Toronto.

Brenner S., Horne R.W. (1959). A negative staining method for high resolution electron microscopy of viruses. *Biochim. Biophys. Acta.* **34**, 103-110.

Brock Th.D. (1988). Robert Koch: a life in medicine and bacteriology. Scientific revolutionaries: a bibliographical series, Science Tech Publishers, Madison and Springer-Verlag, Berlin-Heidelberg.

Brumfield G. (2003). Still out in the cold. *Nature.* **423**, 678-680.

CDC. (2014). Website of the Centers for Disease Control and Prevention. http://emergency.cdc.gov/agent/smallpox/lab-testing/pdf/EM-rash-p.pdf accessed January **19**, 201.

Curry A., Appleton H., Dowsett B. (2006). Application of transmission electron microscopy to the clinical study of viral and bacterial infections: present and future. *Micron.* **37**, 91-106.

Curry A. (2003). Electron microscopy and the investigation of new infectious diseases. *Inter. J. Infect. Dis.* **7**, 251-258.

Czerny C.-P., Eis-Hübinger A.M., Mayr, A., Schneweis, K.E., Pfeiff, B. (1991). Animal poxviruses transmitted from cat to man: current event with lethal end. *J. Vet. Med. B.* **38**, 421-431.

Dane D.S., Cameron C.H., Briggs M. (1970). Virus-like particles in serum of patients with Australia-antigen-associated hepatitis. *Lancet.* **1**, 695-698.

De Carlo S., El-Bez C., Alvarez-Rúa C., Borge J., Dubochet J. (2002). Cryo-negative staining reduces electron-beam sensitivity of vitrified biological particles. *J. Struct. Biol.* **138**, 216-226.

De Carlo S., Harris J.R. (2011). Negative staining and cryo-negative staining of macromolecules and viruses for TEM. *Micron.* **42**, 117-131.

De Haro T., Furness. (2012). Current and future delivery of diagnostic electron microscopy in the UK: result of a national survey. *J. Clin. Pathol.* **65**, 357-361.

Feinstone S.M., Kapikian A.Z., Purcell R.H. (1973). Hepatitis A: detection by immune electron microscopy of a virus-like antigen associated with acute illness. *Science*. **182**, 1026-1028.

Fenner F., Henderson D.A., Arita, I., Jezek, J., Ladny, L.D. (1988). Smallpox and its Eradication. Geneva, *WHO*.

Field A.M. (1982). Diagnostic virology using electron microscopic techniques. *Advanc. Virus Res.* **27**, 2-69.

Flewett T.H. (1985). Rapid diagnosis of virus diseases. *Brit. Med. Bull.* **41**, 315-321.

Flewett T.H., Bryden A.S., Davis H.A. (1973). Virus particles in gastroenteritis. *Lancet.* **2**, 1947.

Freundlich M.M. (1963). Origin of the electron microscope. *Science.* **142**, 185-188.

Gelderblom H.R. (2001). Electron microscopy in diagnostic virology. *BIOforum international.* **5**, 64- 67.

Gelderblom H.R. (2003). Elektronenmikroskopie im Methodenspektrum der Bioterrorismus-Diagnostik. *Bundesgesundheitsbl., Gesundheitsforsch. Gesundheitsschutz.* **46**, 984-988.

Gelderblom H., Bauer H., Ogura H., Wigand R., Fischer A.B. (1974). Detection of oncornavirus-like particles in HeLa cells. Fine structure and comparative morphological classification. *Intern. J. Cancer.* 13, 246-253.

Gelderblom H.R., Schwarz H. (1974). Relationship between the Mason-Pfizer monkey virus and HeLa virus. Immunoelectron microscopy. *J. Natl. Cancer Inst.* **56**, 635-638.

Gelderblom H.R., Reupke H. (1978). Rapid viral diagnosis using the airfuge. Abstract of the Fourth International Congress for Virology. In: *International Virology IV*, The Hague, Netherlands. 630.

Gelderblom H.R., Kocks C., Lâge-Stehr J., Reupke, H. (1985). Comparative immuno-electron microscopy with monoclonal antibodies on yellow fever virus infected cells: pre-embedding labelling versus immunocryoultramicrotomy. *J. Virol. Meth.* **10**, 225-239.

Gelderblom H., Pauli G. (1986). LAV/HTLV-III: Vergleich mit anderen Retroviren und Einordnung in die Subfamilie der Lentivirinae. *AIDS-Forsch.* **1**, 61-72.

Gelderblom H.R., Hausmann E.H.S., Özel M., Pauli G., Koch M.A. (1987). Fine structure of human immunodeficiency virus (HIV) and immunolocalization of structural proteins. *Virol.* **156**, 171-176.

Gelderblom H.R., Renz H., Özel M. (1991). Negative staining in diagnostic virology. *Micr. Microsc. Acta.* **22**, 435-447.

Gelderblom H.R., Hazelton P.R. (2000). Specimen collection for electron microscopy. *Emerg. Infect. Dis.* **6**, 433-434.

Gelderblom H.R., Bannert N., Muss W., Madeley C.R. (2006). Routine, rapid, noninvasive diagnosis of viral skin exanthems. *British J. Dermatol.* **154**, 558-560.

Gelderblom H.R., Bannert N., Pauli G. (2007). Ar-

guments pro disinfection in diagnostic electron microscopy: a response to Madeley and Biel. *J. Infect*. **54**, 307-308.

Gelderblom H.R., Krüger D.H. (2014). Helmut Ruska (1908-1973). His role in the evolution of electron microscopy in the life sciences, and especially virology. *Adv. in Imag. Elect. Physics*. **182**, 1-94.

Gelmetti D., Fabbi M., Sironi G., Grilli G., Lavazza A. (1996). Identification of parvovirus-like particles associated with three outbreaks of mortality in young pheasants. *J. Vet. Invest.* **8**, 108-112.

Gentile M., Gelderblom H.R. (2005). Rapid viral diagnosis: role of electron microscopy. *New Microbiol.* **28**, 1-12.

Gentile M., Adrian T., Scheidler A., Ewald M., Dianzani F., Pauli G., Gelderblom H.R. (1994). Determination of the size of HIV using adenovirus type 2 as an internal length marker. *J. Virol. Meth.* **48**, 43-52.

Gewin V. (2003). Bioterrorism: agriculture shock. *Nature.* **241**, 106-108.

Goldsmith C.S., Miller S.E. (2009). Modern uses of electron microscopy for detection of viruses. *Clin. Microbiol. Rev.* **22**, 552-563.

Goldsmith C.S., Tatti K.M., Ksiazek T.G., Rollin P.E., Comer J.A., Lee W.W., Rota P.A., BAnkamp B., Bellini W.J., Zaki S.R. (2004). Ultrastructural characterization of SARS coronavirus. *Emerg. Infect. Dis.* **10**, 320-326.

Goldsmith C.S., Ksiazek T.G., Rollin P.E., Comer J.A., Nicholson W.L., Teresa C.T., Peret Erdman D.D., Bellini W.J., Harcourt B.H., Rota P. A., Bhatnagar J., Bowen M.D., Erickson B.R., McMullan L.K., Nichol S.T., Wun-Ju Shieh Paddock C.D., Zaki S.R. (2013) Cell culture and electron microscopy for identifying viruses in diseases of unknown cause. *Emerg. Infect. Dis.* **19**, 864-869.

Haguenau F., Hawkes P.W., Hutchinson J.L., Satiat-Jeunemaitre B., Simon G.T., Williams D.B. (2003). Key events in the history of electron microscopy. *Micr. Microan.* **9**, 96-138.

Haguenau F., Hakwes P.W., Hutchinson J.L., Satait-Jeunemaitre B., Simon G.T., Williams D.D. (2003). Key events in the history of electron microscopy. *Micr. Microan.* **2**, 269-271.

Hammond G.W., Hazelton P.R., Chuang I., Klisko B. (1981). Improved detection of viruses by electron microscopy after direct ultracentrifuge preparation of specimens. *J. Clin. Microbiol.* **14**, 210-221.

Harris J.R. (1997). Negative staining and cryo-electron microscopy. Bios Scientific Publications Oxford.

Hawkes P.W. (1985). The beginning of electron microscopy. In: P.W. Hawkes, ed. Advances in electronics and electron physics. Suppl. 16, pag ii, Academic Press Orlando.

Hayat, M.A., Miller S.S. (1990). Negative Staining. McGraw-Hill Publishing Company.

Hazelton P.R., Gelderblom H.R. (2003). Electron microscopy for rapid diagnosis of infectious agents in emergent situations. *Emerg. Infect. Dis.* **9**, 294-303.

Henderson R., Sali A., Baker M.l., Carragher B., Devkota B., Downing K.H., Egelman E.H., Feng Z., Frank J., Grigorieff N., Jiang W., Ludtke S.J., Medalia O., Penczek P.A., Rosenthal P.B., Rossmann M.G., Schmid M.F., Schröder G.F., Steven A.C., Stokes D.L., Westbrook .D., Wriggers W., Yang H., Young J., Berman H.M., Chiu W., Kleywegt G.J., Lawson C.L. (2012.) Outcome of the first electron microscopy validation task force meeting. *Structure.* **20**, 205-214.

Hooke R. (1665). Preface: Micrographia: or some physiological descriptions of minute bodies made by magnifying glasses, with observations and inquiries thereupon. Printed by John Mortyn and James Allestry, London.

Horne R.W. (1974) Virus Structure. Academic Press, New York London.

Hyatt A.D., Zaki S.R., Goldsmith C.S., Wise T.G., Hengsberger S.G. (2001). Ultrastructure of Hendra virus and Nipah virus within cultured cells and host animals. *Microbes and Infection.* **3**, 297-306.

ICTV: Ninth Report of the International Committee on Taxonomy of Viruses. (2011). Editors: King, A.M.Q., Adams M.J., Carstens M.B., Lefkowitz E.J., Academic Press, San Diego, London.

Ivanovski D. (1892). Über die Mosaikkrankheit der Tabakpflanze. Bulletin of the Imperial Academy of Sciences, St. Petersburg, **35**, 67-70.

Jernigan D.B., Raghunathan P.L., Bell B.P., Brechner R., Bresnitz E.A., Butler J.C., Cetron M., Cohen M., Doyle T., Fischer M., Greene C., Griffith K.S., Guarner J., Hadler J.L., Ayslett J.A., Meyer L., Petersen L.R., Phillips M., Pinner R., Popovic T., Quinn C.P., Eefhuis J., Reissman D., Rosenstein N., Schuchat A., Shieh W.J., Siegal L., Swerdlow D.L., Tenover F.C., Traeger M., Ward J.W., Weisfuse I., Wiersma S., Yeskey K., Zaki S., Ashford D.A., Perkins B.A., Ostroff S., Hughes J., Fleming D., Koplan J.P., Gerberding; National Anthrax Epidemiologic Investigation Team. (2002). Investigation of bioterrorism-related Anthrax, Unites States, 2001: epidemiologic findings. *Emerg. Infect. Dis.* **8**, 1019-1028.

Johnsen C.K., Böttiger B., Blom J. (2006). Confirmation of electron microscopic results by direct testing of viruses adhered to grids using nucleic acid amplification techniques. *J. Virol. Meth.* **134**, (1-2), 92-98.

Kapikian A.Z., Wyatt R.G., Dolin R. (1972). Visualization by immune electron microscopy of a 27 nm particle associated with acute infectious non-bacterial gastroenteritis. *J. Virol.* **10**, 1075-1081.

Kausche G.A., Pfankuch E., Ruska H. (1939). Die

Sichtbarmachung von pflanzlichem Virus im Übermikroskop. *Naturwissenschaften.* **27**, 292-299.

KNOLL M., RUSKA E. (1932). Beitrag zur Elektronenoptik, I und II. *Annalen der Physik.* **12**, 607-661.

KOCH R. (1876). Die Aetiologie der Milzbrandkrankheit, begründet auf die Entwicklungsgeschichte des Bacillus Anthracis. *Beiträge zur Biologie der Pflanzen.* **2**, 277-310.

KOCH R. (1877). Verfahren zur Untersuchung, zum Konservieren und Photographieren der Bakterien. *Beiträge zur Biologie der Pflanzen.* **2**, 399-434.

KOCH R. (1882). Die Aetiologie der Tuberculose. *Berl. Klin. Wochenschr.* **19**, 287-296.

KRUEGER D.H., SCHNECK P., GELDERBLOM H.R. (2000). Helmut Ruska and the visualization of viruses. *Lancet.* **355**, 1713-1717.

KSIAZEK T.G., ERDMAN D., GOLDSMITH C.S., ZAKI S.R., PERET T., EMERY S., ET AL. (2003). A novel coronavirus associated with a severe respiratory syndrome. *N. Engl. J. Med.* **348**, 1953-1966.

KURTH A., NITSCHE A. (2007). Fast and reliable diagnostic methods for the detection of human poxvirus infections. *Fut. Med.* **2**, 467-479.

KURTH A., ACHENBACH J., MILLER L., MACKAY I.M., PAULI G., NITSCHE A. (2008). Orthopoxvirus detection in environmental specimens during suspected bioterror attacks: inhibitory influences of common household products. *Appl. Environm. Microbiol.* **1**, 32-37.

LANE H.C., LA MONTAGNE J., FAUCI A.S. (2001). Bioterrorism: a clear and present danger. *Nat. Med.* **7**, 1271-1273.

LA SCOLA B., AUDIC S., ROBERT C., JUNGANG L., DE LAMBALLERIE X., DRANCOURT M., BIRTLES R., CLAVERIE J.M., RAOULT D. (2003). A giant virus in amoebae. *Science.* **299**, 2033.

LAUE M., NIEDERWÖHRMEIER B., BANNERT N. (2007). Rapid diagnostic thin section electron microscopy of bacterial endospores. *J. Microbiol. Meth.* **70**, 45-54.

LAUE M., FULDA G. (2013). Rapid and reliable detection of bacterial endospores in environmental samples by diagnostic electron microscopy combined with X-ray microanalysis. *J. Microbiol. Meth.* **94**, 13-21.

LE DUC J.W., JAHRLING P.B. (2001). Strenghtening national preparedness for smallpox: an update. *Emerg. Infect. Dis.* **7**, 155-157.

LOEFFLER F. (1884). Untersuchungen über die Bedeutung der Mikroorganismen für die Entstehung der Diphterie beim Menschen, bei der Taube und beim Kalbe. *Mittheilungen aus dem Kaiserlichen Gesundheitsamte.* **2**, 421-499.

LOEFFLER F.A., FROSCH P. (1898). Berichte der Kommission zur Erforschung der Maul- und Klauenseuche bei dem Institut für Infektionskrankheiten in Berlin. *Zentralblatt für Bakteriologie, Parasitenkunde Abteilung I.* **23**, 371-391.

LONG G.W., NOBLE J., MURPHY F.A., HERRMAN K.L., LOURIE B. (1970). Experience with electron microscopy in the differential diagnosis of smallpox. *Appl. Microbiol.* **20**, 497-504.

LUFTIG R.B. (1968) Further studies on the dimensions of viral and protein structures using the catalase crystal internal marker technique. *J. Ultrastruct. Res.* **23**, 178-181.

LWOFF A., HORNE R., AND TOURNIER P. (1962). A system of viruses. *Cold Spring Harbour Symp. Quant. Biol.* **27**, 51-55.

MADELEY C.R. (1995). Viruses associated with acute diarrhoeal disease. In: Principles and practice of clinical virology, 3rd edn. Editors: Zuckermann A.J., Banatvala J.E., Pattison J.R., Chichester, Wiley. 189-227.

MADELEY C.R. (2003). Diagnosing smallpox in possible bioterrorist attack. *Lancet.* **361**, 97-98.

MADELEY C.R., BIEL S.S. (2006). For debate: is disinfection of specimens, which may contain unknown or bio-terrorist organisms, essential before electron microscopic examination? *J. Infect.* **53**, 70-74.

MADELEY C.R., FIELD A.M. (1988). Virus Morphology. Second edition, Churchill Livingston, Edinburgh London.

MAST J., DEMEESTERE L. (2009). Electron tomography of negatively stained complex viruses: application in their diagnosis. *Diagn. Pathol.* **4**, 5.

MAUNSBACH A.B., AFZELIUS B.A. (1999). Biomedical Electron Microscopy, Academic Press, San Diego London.

MAYER A. (1886). Ueber die Mosaikkrankheit des Tabaks. *Die landwirtschaftlichen Versuchs-Stationen.* **32**, 451-467.

MILLER S.E. (1986). Detection and identification of viruses by electron microscopy. *J. Electr. Microsc. Tech.* **4**, 265-301.

MILLER S.E., HOWELL D.N. (1997). Concerted use of imunologic and ultrastructural analyses in diagnostic medicine: immunoelectron microscopy and correlative microscopy. *Immunol. Invest.* **26**, 29-38.

MILLER S. (2003). Bioterrorism and electron microscopic differentiation of poxviruses from herpesviruses: dos and don'ts. *Ultrastruct. Pathol.* **27**, 133-140.

MORENS D.M., FOLKERS G.K., FAUCI A.S. (2004). The challenge of emerging and re-emerging infectious diseases. *Nature*. **430**, 242-249.

MURRAY K., SELLECK P., HOOPER P., HYATT A., GOULD A., GLEESON L., WESTBURY H., HILEY L., SELVEY L., RODWELL B., KETTERER P. (1995). A morbillivirus that caused fatal disease in horses and humans. *Science.* **268**, 94-97.

NAGLER F.P.O., RAKE G. (1948). The use of electron microscopy in diagnosis of variola, vaccinia and varicella. *J. Bacteriol.* **55**, 45-51.

Nitsche A., Gelderblom H.R., Eisendle K., Romani N., Pauli G. (2007). Pitfalls in diagnosing human poxvirus infections. *J. Clin. Virol.* **38**, 165-168.

Palmer E.L., Martin M.L. (1988) Electron Microscopy in Viral Diagnosis. CRC Press Boca Raton Peters D., Nielsen G., Bayer M.E. (1962). Variola: Die Zuverlässigkeit der elektronenmikroskopischen Schnelldiagnostik. *Deuts. Med. Wochenschr.* **87**, 2240-2246.

Rake G., Blank H., Nagler F.P.O., McNair, S.T.F. (1948). The relationship of varicella and herpes zoster: electron microscopic studies. *J. Bacteriol.* **56**, 293-303.

Reed K.D., Melski J.W., Graham M.B., Regnery R.L., Sotir M.J., Wegner M.V., Kazmierczak J.J., James J., Stratman E.J., Li Y., Fairley, J.A., Swain, G.R., Olson, V.A., Sargent, E.K., Kehl, S.C., Frace, M.A., Kline, R., Foldy, S.L., Davis, J.P., Damon, I.K. (2004). The detection of monkeypox in humans in the Western hemisphere. *N. Engl. J. Med.* **350**, 342-350.

Roingeard P. (2008). Viral detection by electron microscopy: past, present and future. *Biol. Cell.* **100**, 491-501.

Rossmann M. (2013). Structure of viruses: a short history. *Quart. Rev. Bioph.* **46**, 133-180.

Ruska E. (1934). Über Fortschritte im Bau und in der Leistung des magnetischen Elektronenmikroskops. *Zeitschr. Phys.* **87**, 580-602.

Ruska E. (1980). The early development of electron lenses and electron microscopy. S. Hirzel, Stuttgart.

Ruska H. (1940). Die Sichtbarmachung der bakteriophagen Lyse im Übermikroskop. *Naturwissensch.* **28**, 45-46.

Ruska H. (1943a). Über das Virus der Varicellen und des Zoster. *Klin. Wochenschr.* **22**, 703-704.

Ruska H. (1943b). Versuch zu einer Ordnung der Virusarten. *Arch. Gesam. Virusforsch.* **2**, 480-498.

Schroeder J., Voekl E., Hofstaedter F. (2001). Ultrastructural telepathology - remote EM-diagnostic via Internet. *Ultrastruct. Pathol.* **25**, 301-307.

Schroeder J., Gelderblom H.R., Hauroeder B., Schmetz C.H., Milios J., Hofstaedter F. (2006). Microwave–assisted tissue processing for same-day EM-diagnosis of potential bioterrorism and pathological samples. *Micron.* **37**, 577-590.

Schwarz H., Riedle I., Sonntag I., Henning U. (1983). Degrees of relatedness of T-even E. coli phages using different or the same receptors and topology of serologically cross-reacting sites. *EMBO J.* **2**, 375-380.

Siebeck R. (1936). Professional assessment. In: Ruska E. (1980). The early development of electron lenses and electron microscopy. S. Hirzel, Stuttgart. 123-125.

Stirling J., Eyden B., Curry A. (2012). Diagnostic electron microscopy: a practical guide to tissue preparation and interpretation. John Wiley & Sons, first edition.

Subramaniam S., Bartesaghi A., Liu J., Bennett A.E., Sougrat R. (2007). Electron tomography of viruses. *Cur. Op. Struct. Biol.* **17**, 596-602.

Tiekotter K.I., Ackermann H.-W. (2012). High-quality virus images obtained by TEM and CCD technology. *J. Virol. Meth.* **159**, 87-92.

Tischer I., Gelderblom H., Vettermann W., Koch M.A. (1982). A very small porcine virus with circular single stranded DNA. *Nature.* **295**, 64-66.

Tyrrell D.A.J., Almeida J. (1967). Direct electron-microscopy of organ cultures for the detection and characterization of viruses. *Arch. Virol.* **22**, 417-425.

van Rooyen C.E., Scott M.A. (1948). Smallpox diagnosis with special reference to electron microscopy. *Can. J. Publ. Health.* **39**, 467-477.

von Borries B., Ruska E., Ruska H. (1938a). Elektronenmikroskopische Bakterienaufnahmen. *Wissenschaftl. Veröffentl. Siemens-Werke.* **17**, 107-111.

von Borries B., Ruska E., Ruska H. (1938b). Bakterien und Virus in übermikroskopischer Aufnahme. *Klin. Wochenschr.* **17**, 921-925.

Wright D.M. (2005). Immunoelectron microscope techniques in plant virus diagnosis. *Meth. Mol. Biol.* **295**, 193-206.

Zechmann B., Graggaber G., Zellnig G. (2011). Microwave assisted rapid diagnosis of plant virus diseases by transmission electron microscopy. *J. Visual Experim.* **14**, (56), e2950.

Zhang Y., Hung T., Song J.D., He J.S. (2013). Electron microscopy: essentials for virus structure, morphogenesis ansd rapid diagnosis. *Sci China Life Sci*. **56**, 421-430.

Zheng Y.Z., Webb R., Greenfield P.F. (1996). Improved methods for counting virus and virus-like particles. *J. Virol. Meth.* **62**, 153-159