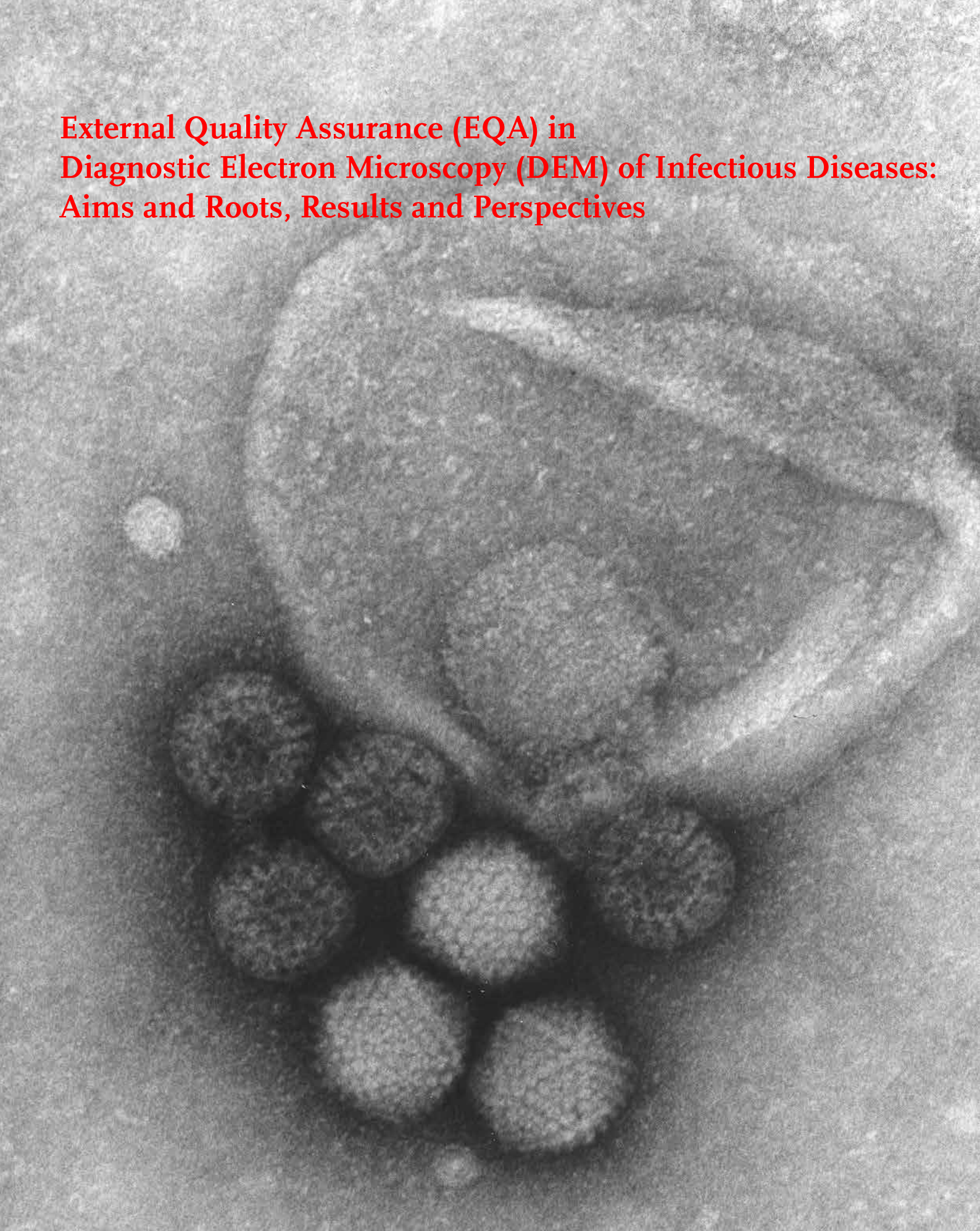
External Quality Assurance (EQA) in Diagnostic Electron Microscopy (DEM) of Infectious Diseases: Aims and Roots, Results and Perspectives



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Prologue - The Microbiome

The human being with its 10^{12} cells is accompanied by some 10^{14} bacteria and still 100-fold more viruses, which together are forming a well-balanced ecosystem. Manifold interactions are taking place between the body and its microbes - in many aspects essential for the human host and well-controlled by its immune system (for a review see c.f. Moelling, 2016). Acute infections and disease arise when the balance of the system is lost due to influences from the outside, e.g., when "new" agents invade an innocent population as exemplified by emerging infections like HIV, Ebola, influenza, malaria, SARS, MERS or monkeypox, just to name a few. Many of them arose or still arise as zoonoses, another reason that human and animal health need to be considered together.

Re-emerging infections, like tuberculosis, measles (e.g., 2015/16 in Berlin) and other communicable diseases controlled in the past, turn up again due to changes in lifestyle and production. Conceptually all infections are emerging or re-emerging diseases (Morens et al., 2004). They are accompanied by the augmentation of the etiological agent followed by an alert of innate and specific branches of the immune defence system. If the immune control is weak or extremely responsive („cytokine storm"), severe disease and even death are caused. To cope with the threats of infections, successful means for disease control like quarantine, laboratory diagnostics and antibiotics were established. Today, basic research and applied health research enable medicine, veterinary medicine and respective health organizations up to the WHO to control many dangerous infections. Nevertheless, the recent re-appearances of Ebola fever 2014/2015 in Central Africa (Carroll et al., 2015) and measles in Berlin reminds us that the fight against infectious diseases will be a never ending task. I. e., considering the potential health threats, the diagnostic and therapeutic armaments against infectious agents must be kept sharp.

Summary

During the 1970s–80s, besides virus culture diagnostic electron microscopy (DEM)[1] became a prime technique in clinical virology and laboratory diagnosis of infectious diseases. Today DEM appears on the retreat for several reasons, e.g., routine DEM is often replaced by molecular, highly sensitive and high throughput methods. Nevertheless, DEM needs to be kept in the diagnostic repertoire to cope with emerging infections, clinical emergencies, quality control (QC), and potential bioterrorism (Biel and Gelderblom, 1999 a; Curry et al., 2006; Gelderblom, 2003 a, b; Gelderblom, 2012; Gentile and Gelderblom, 2005; Hazelton and Gelderblom, 2003; Miller, 2003). As the consequences of an infection or an attack with biological weapons are amenable to intervention only for some hours, the speed of DEM is an essential advantage (Gentile and Gelderblom, 2014; Biel and Madeley, 2001; Madeley, 2003). Speed and "open view" of DEM allow an early and/or a differential diagnosis and also the diagnosis of multiple and/or unexpected infections down to the family level (Almeida, 1963, 1980; Hazelton and Gelderblom, 2003; Goldsmith and Miller, 2009), occasionally also beyond the family level (Goldsmith, 2014).

As the quality of DEM depends to a great extent on expertise and personal skills, it is essential to run DEM in a quality controlled way and supported by continuous practicing and education (Gelderblom, 2001, PDF). After a brief description of historic aspects of QC in DEM of infectious diseases, of its principles and aims, this review focuses on the external quality assurance scheme in DEM (EQA-EMV) and the system of meetings and DEM lab courses run by the Robert Koch Institute (RKI) in Berlin since 1993. This field is depicted on the wider background of the history of the EM group in Virology, founded in 1971 at the RKI.

Introduction

Human and veterinary infectious diseases are diagnosed today routinely by molecular techniques. Agent-specific nucleic acid detection techniques (NADT) and ELISAs are spotting and/or identifying many pathogens in a highly specific and sensitive way. Despite this progress, the morphological description is still essential in linking a new agent to a specific disease. Likewise the visualization of a putative agent can play a key role in the diagnosis of clinical emergencies or of bioterrorism and biological warfare. The sense of vision allows to directly recognize structural details, put them into contexts and derive a rapid, orienting, preliminary or in many clinical cases, a sufficient final diagnosis of the underlying agent.

The direct link between "inspection" and "diagnosis" was held true already in early medicine, e.g., in uroscopy executed in ancient Egypt and used in Europe still during the Middle Ages. The introduction of light microscopy widened the sense of vision down to small, hitherto unvisible items. Using single lens microscopes, Antonie van Leeuwenhoek (1632–1723) described for the first time bacteria and spermatozoa (summarized in Kruger et al., 2000). The instrument was met with great expectations: "... by the help of the microscopes, there is nothing so small, as to escape our inquiry" stated Robert Hooke (1635–1703) in the preface to his MICROGRAPHIA (1665). Systematic studies on the role of bacteria, however, became feasible only in the nineteenth century with the further progress in light optics. In his studies on anthrax, Robert Koch (1843–1910) combined for the first time advanced light microscopy, the isolation of the agent using artificial media, and the reproduction of the disease in an experimental animal (Koch, 1876). The trio of laboratory techniques, later named the three Koch's postulates, also the Koch-Henle postulates (Loeffler, 1884; Brock, 1988) enabled to link an observed or isolated germ etiologically with a particular disease.

In the expectation to trace all communicable diseases back etiologically to specific bacteria, bacteriology flourished at the end of the nineteenth century (Brock, 1988; Kruger et al., 2000). This hope, however, vanished after only three decades when studies on the cause of tobacco mosaic disease and on food-and-mouth disease (FMD) revealed the existence of transmissible agents much smaller than bacteria and therefore not detectable by light microscopy, not hold back by bacterial-tight filters and not growing on artificial media. FMD turned out infectious even after 10.000-fold dilution of the blister fluids from a diseased animal, but there was

1 A list of abbreviations is available at the end of the text

no agent visible even in the best light microscopes (Loeffler and Frosch, 1898). As respective observations were obtained also in studies on tobacco mosaic disease (Beijerinck, „contagium vivum fluidum“, 1898; Ivanovski, 1892), these negatively defined pathogens were summarized soon under the common term “virus”.

The Rise of Electron Microscopy

Apparently, new ways of imaging were required to visualize viruses. The progress was accomplished another three decades later when electron microscopy (EM) was developed during the 1930s, a process taking place mainly in Berlin by three independent groups of electrical engineers (for details see c.f. Haguenau et al., 2003; Ruska, E., 1980; Wolpers, 1991). Using accelerated electrons, characterized by a 10.000-fold shorter wave length compared to visible light, and strong electromagnetic lenses to focus them in a two-step imaging mode, poxviruses were visualized as slightly angular elementary bodies, i.e. the first images of viruses ever published (von Borries et al., 1938). Soon plant viruses, like tobacco mosaic virus (TMV), phages and herpesviruses were studied (summarized in: Gelderblom and Krüger, 2014). Helmut Ruska (1908–1973), physician and younger brother of the 1986 Nobel prize in Physics winner Ernst Ruska (1906–1988), early on interested in the application of EM, established the causal relations of different herpesvirus infections (Ruska, H., 1943 a). Aggregating the available structural and clinico-pathological knowledge he created also the first scientific virus classification (Ruska, H., 1943 b; Kruger et al., 2000).

Due to World War II, the thriving role of German life science EM vanished, while it grew in particular in the US (summarized in: Gelderblom and Krüger, 2014; Rasmussen, 1997). On occasion of the 1947 smallpox outbreak in New York, DEM was applied for the first time as a frontline method in the differential diagnosis of smallpox. This febrile vesicular rash disease in the 1940s and 50s still caused most feared global outbreaks (Ruska, H., 1943 a; Nagler and Rake, 1948; Rake et al., 1948; for a comprehensive review on smallpox, its history and eradication see: Fenner et al., 1988). Similar pioneering DEM was performed by van Rooyen and Scott in Canada (1948). Differences in size and shape of the agents detected in the blister fluids facilitated the necessary rapid differentiation between smallpox and chickenpox/shingles, the latter being caused by the varicella-zoster herpesvirus.

For near two decades, DEM remained confined to the rapid differential diagnosis of smallpox in skin lesions (Williams et al., 1962; Peters et al., 1962; Nagington, 1964; Cruickshank et al., 1966; Long et al., 1970; Sasse and Gelderblom, 2015). Only during the 1970s, concomitant with much ongoing progress in virology and when more convenient to use EM instruments became available, DEM expanded. The morphological peculiarities of a “new agent” helped to classify it, helped to form different virus families (Ruska, H., 1943 b; Almeida, 1963; Lwoff et al., 1962; for a review see c.f.: Gelderblom and Krüger, 2014) and to allocate an observed particle to a specific virus family. With the introduction of the technically simple negative stain preparation (Brenner and Horne, 1959; De Carlo and Harris, 2011; Harris, 1997; Hayat and Miller, 1990), the application of DEM grew considerably. DEM became a “catch-all-method”, fashionable in virology as an almost universal tool in diagnostics and in research. Like the ultracentrifuge, a “must have” for virologists at universities and Public Health Institutions. When microscopists ultimately and with great success applied the “clean” instrument also to body excretions, many virus laboratories started to practise DEM (in Germany see: Philipp and Gelderblom, 1995, PDF) and described a wealth of new viruses: astro-, birna-, calici-, rotavirus and other agents (summarized in Almeida, 1980, 1983; Hazelton and Gelderblom, 2003; Flewett, 1985; Gentile and Gelderblom, 2014; Kjeldsberg, 1980; Madeley, 1995; Miller, 1986; Roingeard, 2008; Zhang et al., 2013). Due to the resolving power of DEM of at least 2 nm, even the smallest viruses, i.e. the circoviruses (17 nm in diameter) can be detected (Soike et al., 2004; Tischer et al., 1982). Basically, merely by size and fine structure, the “open view” of DEM enables a diagnosis at the virus family level. With a number of virus families, e.g., Bunyaviridae, Circoviridae, Filoviridae, Herpesviridae, Poxviridae, Reoviridae or Retroviridae a morphological differentiation also beyond the family level, down to the genus level can be achieved (Goldsmith, 2014). Finally, viruses can also be typed rapidly and reliably by immuno-EM, provided that virus-specific antibodies are available (Biel and Gelderblom, 1999 a; Field, 1982; Miller and Howell, 1997). Immuno-EM, besides virus typing, facilitates also the effective particle enrichment (Gelmetti et al., 1996; Lavazza et al., 2015). As sample preparation and evaluation in DEM involve certain critical steps and depend also on the assistance of an experienced and dedicated staff (Almeida, 1983; Gelderblom et al., 1991; Gelderblom, 2001; Hazelton and Gelderblom, 2003) soon the need for quality assurance in DEM became apparent.

The Development of Quality Assessment Schemes in the Medical Laboratory

Both internal and external quality assurance (EQA) measures are required to guarantee correct, standardized and comparable diagnostic results. To establish an EQA program for clinical biochemistry is relatively straightforward as the constituents of interest like glucose, urea, hormones etc., are well-defined, stable molecules and determined by well established and often automated methods. Diagnostic microbiology, in contrast, is handling an abundance of different pathogenic bacteria, parasites, fungi, prions and viruses using a wide variety of often complex diagnostic methods. Therefore, the selection of proper, stable EQA test specimens in microbiology was and is very demanding. I.e. for technical and economical reasons they need to be restricted to the clinically most relevant agents.

Today‘s EQA measures can be traced back to various origins: in the US there are the College of American Pathologists (CAP) and the Clinical Laboratories Improvement Amendments (CLIA), both overseeing human, not veterinary clinical specimens, and a single State Health Department in the veterinary field (Cynthia Goldsmith, CDC Atlanta, 2014, personal communication). There are no general assessment panels available from either CAP or CLIA, but both of these require proficiency testing twice per year, and

different labs have different approaches to this. Assessment schemes have been run early on also in Canada and Australia (Snell et al., 1982). In Germany the Hämometer-Prüfstelle (hemoglobinometer inspecting authority) of the German Society for Internal Medicine started QC in 1936. Widening the EQA program also into microbiology, the institute was named Institut für Standardisierung und Dokumentation im medizinischen Laboratorium (INSTAND e.V.) in 1968. It is providing a widely developed, however also costly EQA program in different laboratory fields. In Germany, participation in EQA is mandatory for all laboratories involved in human and veterinary health diagnostic activities.

The Origin of EQA in DEM in the UK

In the UK, first EQA trials were organized by the Public Health Laboratory Service (PHLS) in Colindale in 1971. In 1974, following apparent demands, a full-time Microbiology Quality Control Laboratory (MQCL) was established serving all clinical microbiology laboratories by implementing a comprehensive microbiological quality assessment scheme. Enrolment was voluntarily and free of charge (until 1992) and all laboratories involved in human health diagnostics were encouraged to participate. In 1983, overall 198 groups participated in the different EQA programs of the UK National External Quality Assessment Scheme for Microbiology (NEQAS), 170 in the UK and 28 non-UK participants, mainly in Europe (Snell et al., 1982).

In 1977 the PHLS started its EQA scheme in DEM. The first samples were dispatched on August 31 to about 50 laboratories. Twice a year, usually four simulated diagnostic suspensions were distributed to medical laboratories. The specimens were fecal samples or skin scrapings, urine, supernatants from infected cell cultures, occasionally also vesicle fluids (dried between two glass microscopic slides) or homogenized skin warts (Vivienne James, UK NEQAS, London, 2014, personal communication). The samples contained unfixed, life virus, with one of the four specimens being a negative control, and were accompanied by clinical data and a form to report the DEM results back to NEQAS. Participants were asked also to describe details of their preparation and the size, morphology and state of preservation of the agent observed. In 1983, there were 62 laboratories enrolled in the EQA-DEM program (Reed et al., 1985 a, b). Test specimens covered the range from “small round particles” (SRP) to the more safe to diagnose adeno-, herpes-, ortho- and parapox-, reo- and rota-, and ortho- and paramyxoviruses, occasionally also mixtures of SRP with other well-known viruses (Dick Madeley, Newcastle, UK, 2014, personal communication). Provided the samples were properly stained, the latter did not cause a serious challenge, while some of the SRP agents, ranging from 40 nm in diameter down to less than 20 nm, e.g., picorna-, parvo-, astro-, and caliciviruses, occasionally raised trouble. The reported results were marked by a system of “graded correctness” on a -1 to +2 scale.

Between 1977 and November 1993, a total of 83 samples were sent out. When in the early 1990s high throughput diagnostic tests were introduced, DEM started to lose its fundamental role in routine viral diagnosis. Though there were still 58 laboratories participating in the last two EQA runs in May and November 1993, the interest in the scheme faded: only 52, respectively 50 participants returned their results. The downward trend was reinforced by the fact that the EQA schemes had to finance themselves from the participant´s subscriptions when in 1992 the UK Department of Health withdrew its financial support. Accordingly NEQAS, after 33 distributions, discontinued its EQA-EMV program (Vivienne James, NEQAS London, 2014, personal communication).

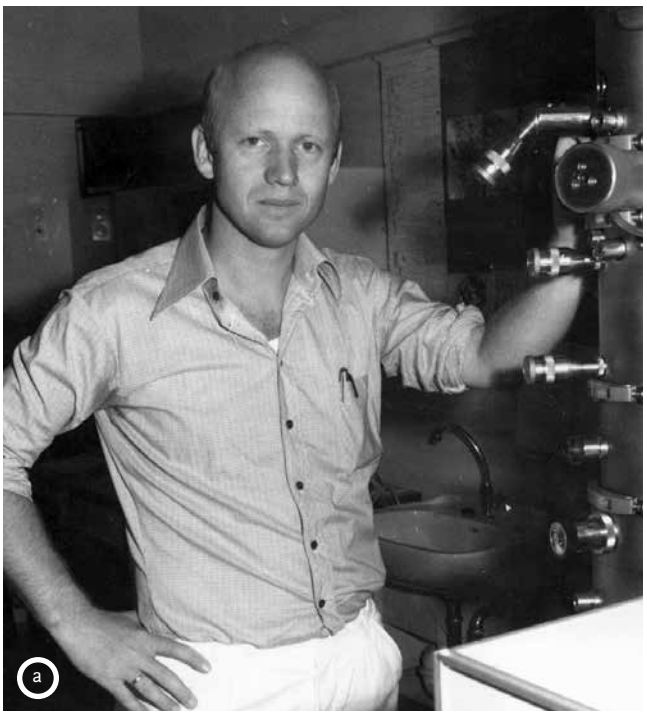


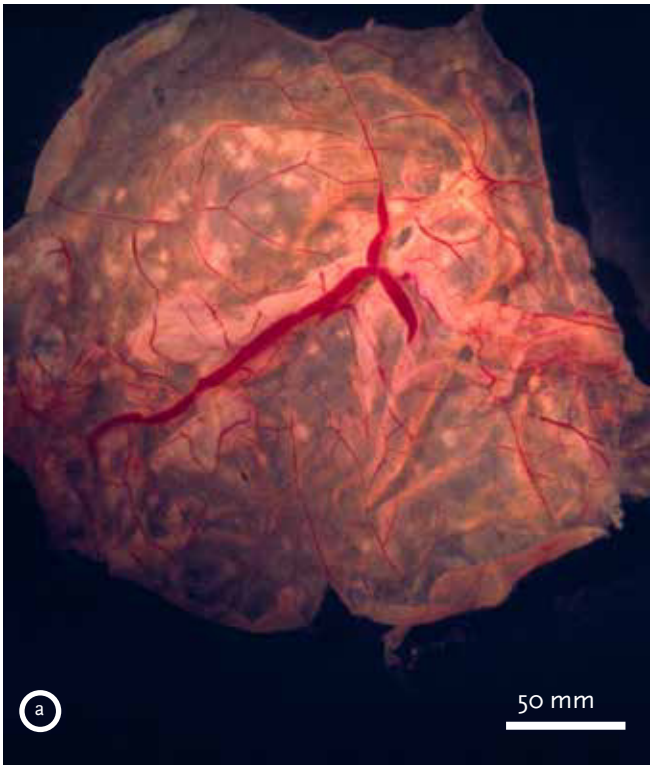
Figure 1: (a) Hans Gelderblom with „his" new Siemens TEM 101 in 1972 forming the core of the EM group in the Virology Department. The formation of this group was very much furthered by Peter Giesbrecht, the head of the Cell Biology Department at the RKI. The TEM was extensively used: 43.855 negatives were taken. It required weekly cleaning, e.g., of the "Putzröhrchen" (a delicate 3 mm small diameter lead-through tube), of the condenser and objective apertures etc., and went out of action in September 1997.

(b) Hannelore Pietruschok, a cleaning lady worked in our group mainly as a lab assistant, e.g., also typewriting the key data on the record cards for our EM negatives. In 1976, when WHO had asked again to eliminate all stocks of variola virus, she was a great help in cleaning up the deep freezers of the RKI. Photos by Anneliese Passow, RKI

The Roots of EQA in DEM at the Robert Koch Institute

Protection against infectious diseases is a central task of the Robert Koch Institute (RKI) in Berlin. It gives direct advice to the Ministry of Health - based on in-house bio-medical research and expertise - and in case of outbreaks offers help by a "rapid task force" to the Federal States, as health issues in Germany are in the direct responsibility of the Federal States. In the Centre for Biological Threats and Special Pathogens (ZBS) of the RKI, established in December 2001 as a consequence of the 9/11 events in the US, measures were developed to respond to particular biological threats like outbreaks of highly dangerous infectious diseases, biowarfare or bioterrorism.

Three decades earlier, by September 1971, the Virology Department of the RKI was reinforced by Karin Mölling, Heinz Bauer, Reinhard Kurth and Hans Gelderblom (hg), a retrovirus-focused crew coming from the Max-Planck -Institute for Virus Research in Tübingen. By July 1974, after only four years this crew, together with Bob Fries, Hajime Ogura, Georg Pauli, and Larry Rohrschneider as additional members, left the RKI for Giessen university, except of Hans Gelderblom. Yet, the medically trained hg had developed links already to the Virchow Campus nearby, to Infectious Diseases (Professor Hans D. Pohle), Dermatology (Professor Günter Stüttgen) and Dentistry (Professor Peter A. Reichart). As smallpox was still smoldering with outbreaks mainly in India and Somalia (Fenner et al., 1988), global travel confronted Berlin repeatedly with cases of suspected smallpox. Therefore, an EM group in Virology was established, headed by Hans Gelderblom (Fig. 1a). All suspected cases turned out "orthopoxvirus-negative", though one patient presented even records from a previous hospitalization in a Smallpox Hospital in India. The last real smallpox case in Germany occurred in Hannover in 1972 (Sasse and Gelderblom, 2015). The orthopoxvirus (OPV) was rapidly visualized by NS-DEM (Höpken et al., 1973). After propagation of the isolate on the chorio-allantoic membrane (CAM) of pathogen-free eggs (Fig. 2a), an abundance of significant OPV-particles was shown also in routine thin section transmission electron microscopy (TS-TEM) (Fig. 2b). As routine embedding and TS-TEM require a time frame of 4–5 days up to the final diagnosis, we applied also rapid embedding techniques using low viscosity resins (Spurr, 1969). Thus, the time to a TS-based diagnosis was shortened to only two days. Nevertheless, because NS is unmatchable rapid and much less dependent on specific skills and man power, we preferred NS-TEM in routine DEM. Poxvirus diagnostics was performed in West-Berlin mainly by the RKI in cooperation with two institutions of the neighbouring Virchow University Clinical Center, either with the Infectious Disease Department and its quarantaine station - then and also today the largest infectious diseases hospital in Berlin, or with the Dermatology Department. NS-DEM of vesicle fluids followed by inactivation of the grids by formaldehyde



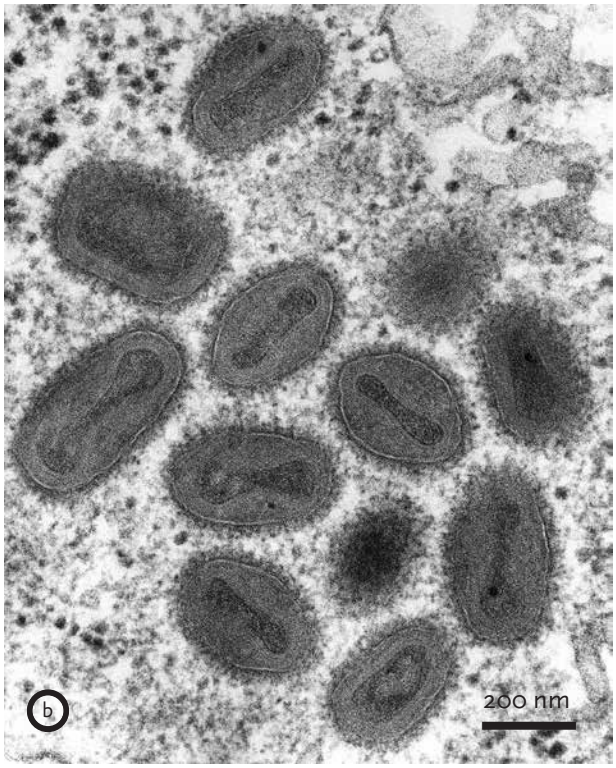


Figure 2: (a) Part of a chorio-allantoic membrane (CAM) inoculated with vesicle material collected from the last smallpox case in Germany, Hannover 1972 (Höpken et al., 1973). The transparent CAM shows blood vessels and small white pocks, often confluent and forming plaques. The pocks are due to massive cell proliferation and pathognomonic for the smallpox virus in contrast to the larger pocks induced on the CAM by vaccinia virus and the herpes simplex and varizella-zoster herpesviruses. Photo by Anneliese Passow, RKI

(b) TS-TEM of a CAM cell in one of the small plaques induced by the Hannover smallpox virus shown in (a) (Epon embedding). Mature OPV particles are sectioned at different angles. Virus assembly and maturation take place in the cytoplasm. The "brick-shaped" particles appear slightly rounded in TS-TEM, measure in two dimensions 350 x 200 nm, and show a complex fine structure (compare c.f. with Fig. 12a). Under a thick outer membrane wall, lateral bodies and the dumbbell-shaped inner body can be differentiated. Photo by hg, RKI

steam was done in parallel to injecting embryonated eggs to isolate any virus on the CAM. Fortunately, in all 12 emergency cases exclusively particles of the herpesvirus family were detected, even from the most smallpox-suspected patients. Most difficult, of course, were two suspected clinical cases, which remained repeatedly "virus-negative" by DEM. No fun, because the consulting offices and the clinic, in demand of a rapid DEM diagnosis, were closed down for several hours for safety reasons.

Finally, urged by WHO and the German Ministry of Health, hg together with Hannelore Pietruschok (Fig. 1b), an always helpful laboratory scrubwoman autoclaved all variola virus stocks of the RKI in January 1976 - fully convinced and without any bad feelings to have supported eradication of an unique species and possibly useful, future research materials. It may be noted here, that some members of the emergency diagnostic staff hated variola also because of the cold biosafety shower after the diagnostic work.

While the Department of Cell Biology of the RKI, led since 1958 by Professor Peter Giesbrecht (1930–2001), applied EM in bacteriology to elucidate basic biological and in particular antibacterial mechanisms (Giesbrecht et al., 1976), the new 1971 EM Group in Virology focused on two fields, (retro-) virus research and clinical virology to diagnose infections directly from the patient (Remy and Gelderblom, 1974; Fig. 3) or from diagnostic cultures and to screen for "new" viruses in body secretions and help establishing in-house ELISAs and other new diagnostic tools. The work benefited from the cooperation with students, mainly biologists and veterinarians interested to do the research required for their diploma or doctoral theses. They stayed often for more than one year and were payed a small income - beneficial conditions considering todays Bachelor and Master study regimes. We hosted also a number of full-time guest scientists. Lectures on bio-medical EM were run at the Free University and connections established to University Hospitals in West-Berlin to get access to specimens and clinical information. By July 01, 1977 the Group "Fine Structure Research in Virology" (Fachgebiet Feinstrukturforschung in der Abteilung Virologie) was officially established. As success did not fail to materialize, we became able to reinforce the staff of the EM Group (Fig. 4), to acquire another TEM, and in 1978 we moved into the new Laboratory Building of the RKI, Nordufer (Fig. 9b).

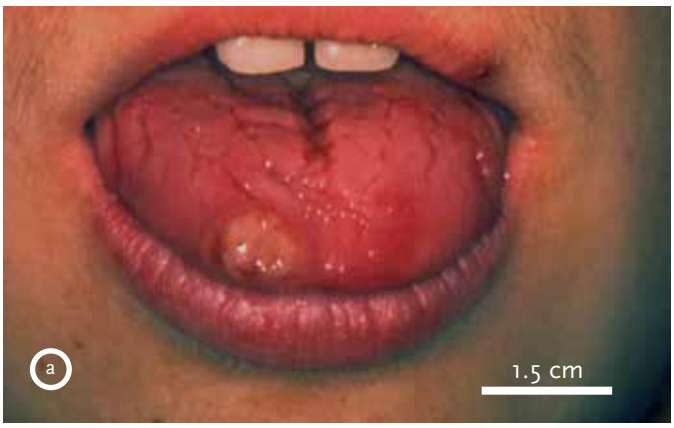
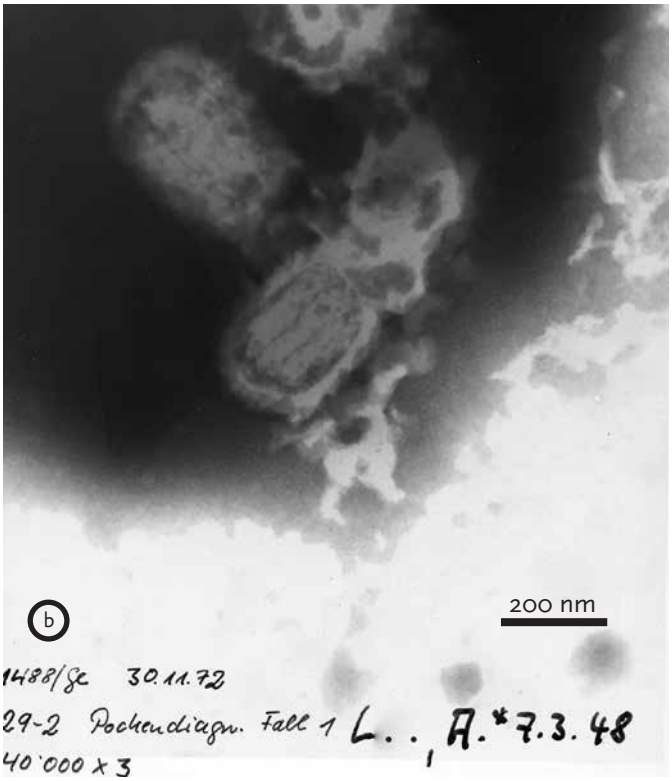


Figure 3: (a) The tip of the tongue of a young mother presenting a whitish lesion 0.6 to 0.8 cm in diameter of a supposed luetic origin. When dark field light microscopy failed to reveal Treponema pallidum bacteria in three attempts, doubts were raised in the Poliklinik and the patient was transferred to the EM group at RKI. Photo by Professor Günter Stüttgen, Berlin



(b) After direct touch preparation from the ulcerous lesion, NS with 2 % PTA and formaldehyde-steam inactivation, brick-shaped particles 400 x 250 nm were visualized instantly, most of them stain-penetrated and thus revealing details of an inner organization. Despite the poor structural preservation, size, shape and the "triple coil", i.e. the inner body are typical for OPV (Peters and Müller, 1963). How come? The 1.5 years old child of the young mother had been vaccinated against smallpox two weeks before the lesion appeared on the mother's tongue. Seeing the coy and decent mother, Professor Stüttgen did not believe in a luetic lesion (in contrast to his staff of young assistant doctors) and send her for DEM. The particles observed made it highly likely that the lesion was caused by a child-to-mother smear transfer of the life vaccine (Remy and Gelderblom, 1974; hg). What can we and can Dermatology learn in addition? With skin lesions there is not always a need for taking biopsies - as stated much later in the British Journal of Dermatology (Gelderblom et al., 2006: unfortunately a badly illustrated article: BJD had ignored all advice for a proper print size of the images). Photo by hg, RKI

By December 09, 1979, WHO declared "that Smallpox has been eradicated from the World" (Fenner et al., 1988). In the official two years smallpox post-eradication period, in 1980–81, more than 4000 dubious febrile vesicular rashes were investigated worldwide. DEM revealed exclusively members of the herpesvirus family, thus ruling out smallpox (Fenner et al., 1988). The WHO declaration, however, was not the end of any poxvirus threats. The world learned soon about poxvirus zoonoses, in particular in immuno-compromized patients (Czerny et al., 1991; Reed et al., 2004; Kurth and Nitsche, 2007; Kurth et al., 2008; Shchelkunov, 2013) and possible bioterror scenarios (Brumfiel, 2003; Lane et al., 2001; Meltzer et al., 2001; LeDuc and Jahrling, 2001; Madeley, 2003; Miller, 2003; Gewin, 2003). Considering also the growing volume of routine DEM in virology, the need for EQA in DEM became apparent (Biel and Gelderblom, 1999 b; Gelderblom et al., 1991; Gelderblom, 2001).

Besides the EQA-EMV scheme in the UK (see above) also an educational program in the former German Democratic Republic (GDR) is to be mentioned here: the Working Group "Electron Microscopy" within the Section "Laboratory Medicine" of the Scientific Veterinary Medicine Society (Arbeitsgemeinschaft Elektronenmikroskopie in der Fachkommission Labordiagnostik der Wissenschaftlichen Gesellschaft für Veterinärmedizin der DDR). Founded in 1977, the Group held annual meetings that were attended by scientists and staff of the veterinary offices located at the 15 area authorities of the former GDR, Universities, and State Research Institutes. A wide field of topics was treated: from rapid NS-DEM to basics in immuno-EM and histopathology of HIV. The last meeting headed by Professor Volker Bergmann from the Veterinary Faculty of the Humboldt-University Berlin was run in Dresden by April 18–19, 1990 and attended by 35 persons. Previous, by November 09, 1989 the Berlin wall came down and after lengthy negotiations, the two Germanies re-united by October 03, 1990. This logic development initiated, however, also a number of harmful economic, organizational and personal changes mainly in the former GDR, so that by December 08, 1990 the Working Group declared its voluntary liquidation (Kathrin Hoffmann, Dresden, and Karl-Friedrich Reckling, Stendal, 2014 personal communications).

Figure 4: (a) The EM Group Fachgebiet 122 in 1985 as part of the Virology Department on the third floor of the Laboratory Building of the RKI at Nordufer 20. The core consisted of three technicians and two scientists, Muhsin Özel (sitting) and Hans Gelderblom (far right). From left to right: Bärbel Jungnickl (photographic assistant), Hannelore Pietruschok (lab-assistant), Thorsten Winkel (student), Regina Scheidler (technical assistant), Hilmar Reupke (technical assistant), Sylvia Pietschmann (student), Holger Wirtz (photographic assistent), Elda Hausmann (student).

(b) The same group after some fluctuations 10 years later in 1995, still in Virology, before the move into the basement and the fusion in 2000 with the Cell Biology Department. Left to right: Deputy Head Muhsin Özel (01.01.1981 – 17.02.2006 at RKI) with his technician Katja Dunckelmann. Standing: the photographic assistant Bärbel Jungnickl (01.02.1982–30.06. 2012 at RKI), sitting Stefan Biel as diploma student, Freya Kaulbars (technician, starting 25.02.1994 on a 25 % position, leaving 31.01.2007), hg (01.09.1971–30.05.2004), the technician Monika Ewaldt and Massimo Gentile, a fellow scientist from Rome. This crew executed research and routine lab work as well as regular outside activities, like track and field, skating, and annual excursions into museums and castles followed by dining wild boar rookie.

Starting EQA in DEM at the Robert Koch Institute

Early in 1993, Hans Gelderblom listed the aims of a future program and approached Professor Dieter Großklaus, the then President of the German Federal Health Office in Berlin for approval and financial support. The aims of the program were:

1 to figure out the use of DEM in Germany in both Medicine and Veterinary Medicine quantitatively and qualitatively in order to define the requirements for its satisfactory performance and how to execute Quality Control (QC).

2 to provide stable virus suspensions for DEM to assess the performance of participants in sample preparation, evaluation and interpretation.

3 to monitor and control the performance of participants in EQA and - where required - encourage participants to improve their performance.

4 to initiate a comprehensive educational program consisting of Scientific and Applied Meetings and Laboratory Training Courses.

5 to identify "weak points" in DEM and develop and promote procedures for a more safe and efficient performance, e.g., "sticky grids", enrichment techniques, the use of detergents, immuno-EM, rapid embedding, biosafety and other measures.

When the project was approved with proper sums to pay for a student and for consumables, the ramifications of the project were discussed in April 1993 with Professor Günther Maass, Head of the German Society against Virus Diseases (Deutsche Vereinigung zur Bekämpfung der Viruskrankheiten e.V., DVV). Decidedly interested, he offered his institutional support. So did colleagues from the former Arbeitskreis Elektronenmikroskopie of the GDR, the Deutsche Gesellschaft für Elektronenmikroskopie (Bernd Tesche, DGE), the Gesellschaft für Virologie, and Professor Heinz Zeichhardt from INSTAND e.V.. Networking helped to promote the EQA program and to recruit participants. A student in Veterinary Medicine, Susanne Philipp, took over the project as part of her doctoral thesis (Philipp, 1996).

Human health was, and still is, institutionally and economically separated from the fields of Veterinary Medicine. This gap may be justified in many aspects, however, not so much in virology and not at all in the DEM of infectious agents. From the start on, and against much opposition, we involved both fields in the EQA-EMV scheme, anticipating already the current ONE HEALTH tendencies.

To determine the current use of DEM and the demand for a respective EQA program in Germany a written survey was undertaken with 471 virus laboratories covering virtually all virus activities in Public Health, universities, industry, and Armed Forces by March 1994. About 68 % of the addressed laboratories responded back, 283 in medicine and 38 in veterinary medicine, reporting besides other data a total annual number of 22.000 DEM specimens in Germany. In veterinary medicine all 14 institutions performing in-house DEM wished to participate, while in medicine 16 of 19 actively performing laboratories were ready to participate (Philipp and Gelderblom, 1995).

What Kind of Test Samples to Use in the EQA-EMV Scheme?

In order to avoid unnecessary economic and administrative stress for both the EQA-participants and for us, the scheme was, and still is, free of any charges. We resorted to either non-purified virus progeny directly from infected cell cultures or stool suspensions, because purified virus is particular labile. Occasionally, also vesicle fluids dried down on glass slides as well as homogenized warts were distributed. All these raw materials contain also biological constituents, amino acids, proteins, sugars known to preserve labile structures. In addition, to counter particle damage due to uncontrolled long shipment, the suspensions were routinely pre-fixed using freshly prepared paraformaldehyde (PFA, 1 up to 4 % final concentration with the addition of merthiolate against bacterial growth, occasionally with the addition of 0.05 % glutaraldehyde, GA). Pre-fixation stabilized indeed and did not induce a loss of particle fine structure nor an appreciable cross-linking of virus particles. Virus aggregates were not observed more often nor increased in size in the control preparations performed six month after pre-fixation (Fig. 6). Fixation served also lab safety, another not neglectable aspect (for a discussion see c.f.: Madeley and Biel, 2006; Gelderblom et al., 2007). A recent study on inactivation of virus suspensions for DEM now is providing a safe and rapid (60 min) protocol using 2 % buffered formaldehyde and a temperature-shift from 25 to 37 °C after 30 min, which is especially useful for emergency cases (Möller et al., 2015).

In order to make the 30 participants acquainted with the kind of suspensions to be supplied later, pre-fabricated, ready-made EM grids were distributed in the first two EQA-EMV runs. Textbooks in virology and atlases (Connor et al., 1997; Dalton and Haguenau, 1973; Doane and Anderson, 1986; Horne, 1974; Hsiung et al., 1994; ICTV, 2011; Laue and Möller, 2015; Lenette et al., 1995; McLean and Wong, 1984; Madeley and Field, 1988; Murphy, 2012; Palmer and Martin, 1988; Rossmann, 2013) serving as references on viral fine structure normally depict well preserved, "ideal" agents in a well-balanced NS layer. In the reality of DEM, however, "atlas images" are not regularly met. The technically robust NS-DEM involves an adsorption and air-drying step (Biel and Gelderblom, 1999 a; Gelderblom, 2003 a; Hazelton and Gelderblom, 2003; Gentile and Gelderblom, 2005), both leading to particle deformation and artefacts. E.g., often virus diameters will increase in DEM due to particle flattening. In additon, virus constituents may be lost, i.e. reo- and rotaviruses may lose their outer capsid layer, corona- and retroviruses may shed surface peplomers and adenoviruses their vertex fibres. After NS, many enveloped viruses, e.g., members of the arena-, bunya-, orthomyxo-, paramyxo- or retroviridae, show besides flattening often grotesque deformation due to surface tension and osmotic damage. Minor degrees of membrane damage result already in increased permeability, i.e. the electron dense "stain" after penetration of the virion will reveal also diagnostically significant internal virus structures. Ultimately, envelopes may rupture or are lost completely, setting free diagnostically significant inner viral components, e.g., the 18 nm ribonucleoprotein strands (RNP) of paramyxoviruses or the 100–110 nm herpesvirus capsids. Fortunately, DEM does not depend on particularly well preserved, intact particles. In contrast, DEM rather often benefits from particle damage!

Ready-made specimen grids were used to monitor the pattern recognition abilities of the participants and to make them used to a state-of-the-art, properly spread negative staining. The first EQA-EMV run of the RKI was dispatched in June 1994 (Philipp, 1996; Philipp and Gelderblom, 1995). It consisted of 10 EM-grids prepared from five different virus suspensions (two grids per suspension): herpes simplex virus (HHV-1), human adenovirus type 2, human rotavirus, and Newcastle disease (paramyxo)virus (NDV). Samples were prepared on 400 mesh square copper grids covered with a Pioloform plastic film, reinforced with a thin carbon layer and contrasted with 2 % phosphotungstic acid (PTA), pH 6.5. Before shipment, every fifth of the grids was checked in-house by EM with regard to its suitability. The grids showed homogeneous stain distribution with the majority of particles one-sidedly embedded in the stain. The applicability of the test grids was assessed also by a panel of four external "reference laboratories" selected by our EQA group.

Figure 5: Letter and sample container of EQA-EMV 26 prepared by Lars Möller in 2000 to show the efficient and inexpensive transport mode using fixed, i.e. inactivated test suspensions.

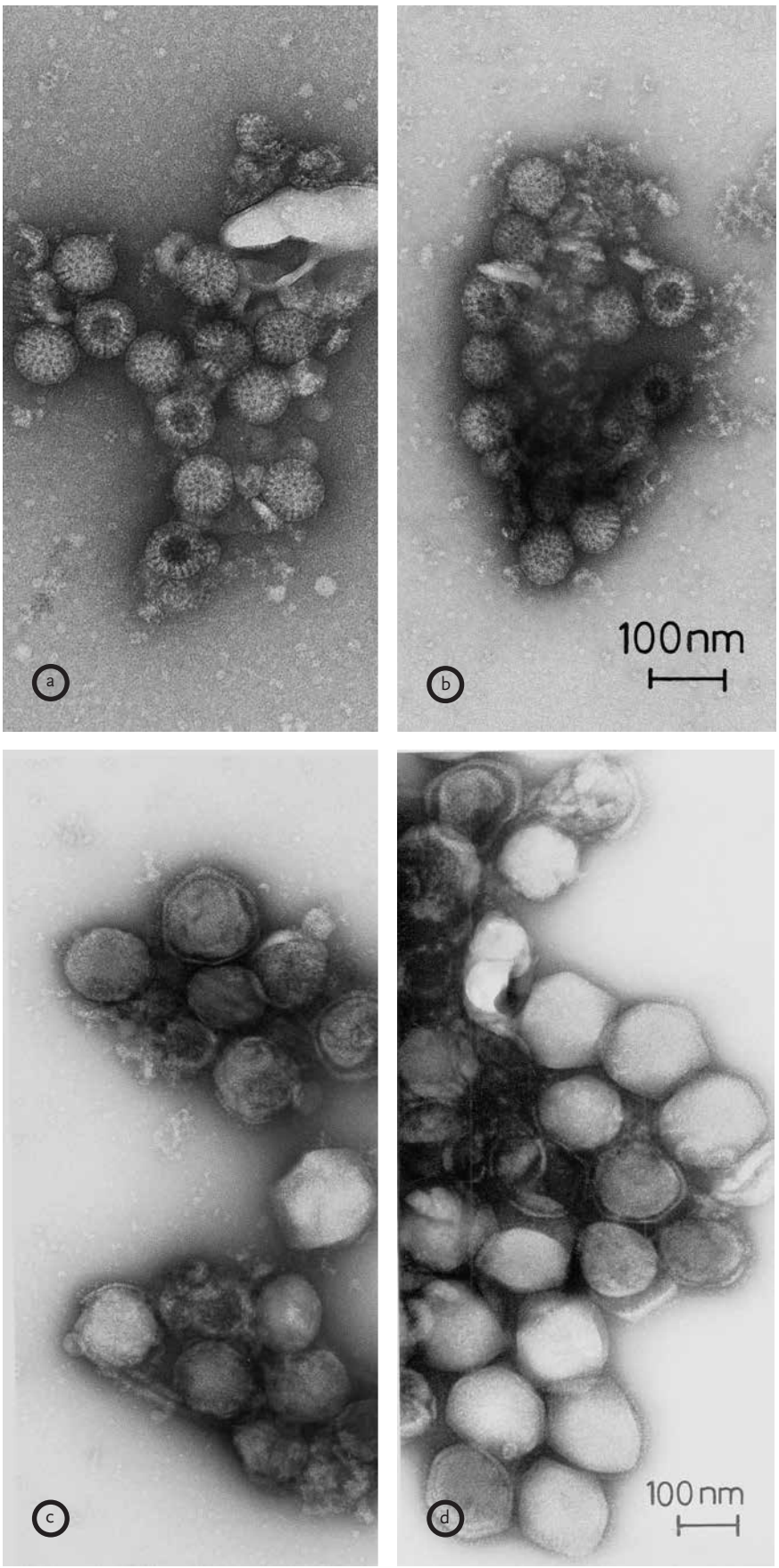


Figure 6: Effect of pre-fixation on stability and fine structure of EQA-EMV samples. (a, b) Sample #5 of EQA-EMV 10, a lysate of simian rotavirus infected cells contrasted with 1 % UAc directly (a) and after five month of storage at 4 °C (b). Both images show besides complete virions also incomplete ones and a few stain-penetrated particles, all of them allowing a clear-cut, rapid DEM of rotaviruses. Photos by Andrea Männel, RKI

(c, d) Sample #4 of EQA-EMV 10: a complex enveloped insect virus (cricket iridovirus) contrasted with 1 % UAc, directly (c) and after five month of storage (d). Also the long-term stored iridovirus sample (d) does not show a loss in fine structure meaning that the prolonged storage of FA-/GA-fixed samples does not impede the diagnostic pattern recognition of iridoviruses. Photos by Andrea Männel, RKI

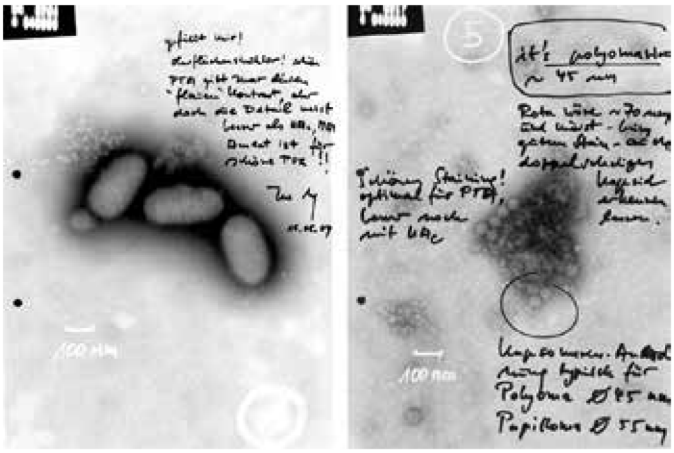


Figure 7: Prints of DEM findings sent back to RKI by a participant in EQA-EMV 16 with the comments and advice of hg regarding quality of preparation and interpretation of the observations.

Results of the EQA-EMV -1 and -2 Runs

Of the 30 participants 28 reported back. "Herpesvirus" was diagnosed correctly by all laboratories, adeno- and rotavirus particles by 25 and 27 participants, respectively. The NDV sample, however, was judged by 20/28 laboratories as an orthomyxovirus specimen, a mis-diagnosis probably due to the high structural stability of the particular NDV and it's relatively to other paramyxoviruses narrow size distribution. Indeed, intact "NDV-bags" are observed not too seldom, in particular after NS with uranyl acetate (UAc). This stain is both, "a stain and a fixative" (Terzakis, 1968). By the way: as enveloped viruses acquire their lipid containing outer layers by budding from cellular membranes they can reveal wide differences in membrane stability reflecting the membrane constituents and properties of their particular host cell (Gelderblom et al., 1987b; Gelderblom, unpublished). The NDV-grids did not show any "free" RNP strands, which are routinely found with more labile paramyxovirus species, e.g., with measles or Sendai virus. The negative control specimen was correctly recognized by 26/28 laboratories. Closed viral "bags" can be seen also with herpesviruses. When NS with the membrane-destabilizing PTA and/or repeated washing on droplets of distilled water before NS did not liberate the viral RNP, a brief incubation of the grid with adhering virus on a droplet of NP-40 detergent (0.1 % in distilled water) was recommended by our lab.

The second EQA distribution was sent out in October 1994 to 28 participants. It consisted of three ready-made grids [UAc-stained Sendai- (paramyxo-), vesicular stomatitis (rhabdo) virus (VSV), and a negative control] and three inactivated suspensions (herpes-, paramyxovirus, and a negative control). The results of the pre-fabricated grids: 24/28 laboratories diagnosed the paramyxovirus correctly, while two laboratories reported orthomyxoviruses and one each filo- and rhabdovirus particles. The negative control and the VSV specimen were correctly assessed by all participants. When the participants prepared the suspensions using their own DEM grids, 27/28 laboratories attained the correct diagnosis "particles of the herpesvirus family", but only 11/28 the correct diagnosis of paramyxovirus particles. The negative control was detected correctly by 24/28 participants.

Later distributions have been described briefly (Möller et al., 2012, PDF). They consisted usually of six virus suspensions, distributed twice a year, in spring and in autumn (Table 1). After pre-fixation and a quality control check of the test suspensions, a volume of 200 or 250 microliter was filled in 0.5 ml plastic tubes (Eppendorf safe lock), packed into a plastic container (Sarstedt, Germany) into cushioned envelopes together with an accompanying instruction and result form and mailed by regular letter service (Fig.5). The letter commented details of the preceding EQA run: a discussion of the reported diagnoses and flaws, recommendations how to improve preparation and assessment, a calendar of the forthcoming educational events and "clinical information" on the enclosed samples. Participants were also encouraged to submit images documenting their results, which provided an opportunity to detailed comments (Fig.7).

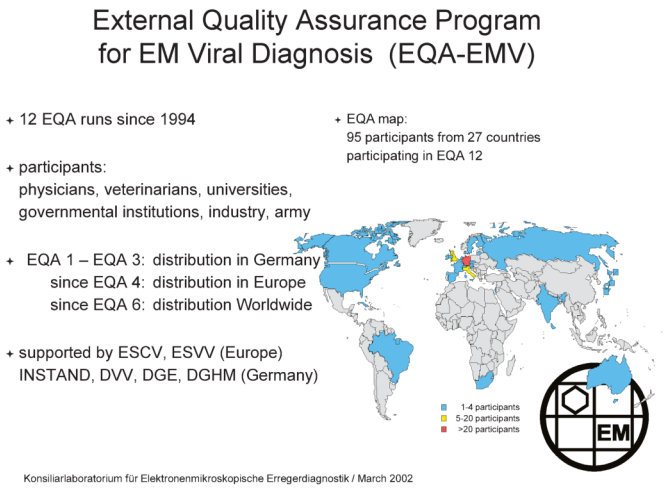


Figure 8: Map showing the regional distribution of the participants of the EQA-EMV 12 sent out February 2002.

Expanding the EQA-EMV Scheme

The first three EQA distributions involved DEM laboratories in Germany only. Starting with EQA-EMV 4, by September 1996, also laboratories from all over Europe enrolled with the strong moral support by the European Society for Clinical Virology (ESCV). As the Chairman of ESCV stated: „this program is of great interest, and it is a very valuable addition to the ESCV program [...] will be an important factor in assurance of high quality in a small but important field of diagnostic virology" (Miklos Degré, letter to the Robert Koch Institute, February 27, 1998). The growth of EQA-EMV in Europe and its further global development are documented in Table 1 and in Figs. 8 and 10.

Going Europe and widening the volume of the EQA-program received ambitious support also internally. Stefan S. Biel, a student in biotechnology, joined our group initially in 1994 to do his Student's Work and later his Thesis. Stefan Biel assisted greatly in the further development of the EQA-EMV scheme. A number of basic publications on the methods of DEM emerged (Biel and Gelderblom, 1999 a, b; Biel and Madeley, 2001) giving rise also to further pertinent papers (Biel et al., 1999, 2004). In 2000, his work was acknowledged by the INSTAND award for diagnostic achievements (Fig. 9).

Table 1. Overview of RKI's EQA-EMV distributions: date, number of test sets sent out, number of countries participating, response rate and percentage of correct diagnoses

EQA-EMV no.	date of distribution	no of recipients	no of countries	response rate [%]	[%] of correct diagnoses
1	Jun 1994	30	1	93.3	72.1
2	Sep 1994	28	1	100	72.0
3	Dec 1995	34	1	100	41.7
4	Sep 1996	35	3	100	56.4
5	Jun 1997	50	10	98.0	70.7
6	Feb 1998	57	16	98.2	73.0
7	Mar 1999	75	18	77.3	72.5
8	Dec 1999	87	21	66.7	65.1
9	Aug 2000	94	22	71.3	67.4
10	Jan 2001	89	23	76.4	67.6
11	Nov 2001	88	23	75.0	80.3
12	Feb 2002	95	25	78.9	62.7
13	Sep 2002	91	25	85.7	79.8
14	Mar 2003	97	23	81.4	79.5
15	Oct 2003	101	26	87.1	66.0
16	Apr 2004	112	26	75.9	76.2
17	Nov 2004	112	28	82.1	70.9
18	Apr 2005	115	27	78.3	89.6
19	Mar 2006	116	22	66.4	64.2
20	Jun 2007	115	25	74.8	65.5
21	Jul 2008	106	27	80.2	79.2
22	Jul 2009	103	26	83.5	83.9
23	Aug 2010	107	26	73.8	80.6
24	Sep 2011	103	24	79.6	67.1
25	Oct 2012	105	22	74.3	68.6
26	Nov 2013	92	25	85.9	85.0
27	Oct 2014	96	27	82.3	80.1
28	Jan 2016	95	30	87.4	79.5

Figure 9: (a) Stefan Biel, hg and Freya Kaulbars - left to right - in the "in-house garden" of the laboratory building of the RKI in September 2000. Stefan contributed much to the Europe - wide growth of the EQA-EMV distributions while the technician Freya Kaulbars performed mainly TS-TEM controls of EQA-EMV samples, besides her main tasks in embedding and TS-TEM in basic virology. Freya stayed from February 1994 to January 2011 with our group. Photo by Hans-Günter Bredow, RKI

(b) The former laboratory building (silver) accomodating the EM group in Virology on the third level, since 1999 in the basement. The Consulting Lab is an integral part of the EM group Fachgebiet ZBS4. Photo by Andrea Schnartendorff, RKI

Also participants from nations outside Europe enroled in the EQA-EMV and participated frequently also in respective educational programs. After 9/11, 2001, when biosafety preparedness became also officially an issue, further DEM laboratories were delegated by their respective Governments as future lead laboratories in their countries. E.g., Argentina, Brasil, Canada, China, India, Japan, Malaysia, Singapore, Mexico, Russia (Novosibirsk region), USA, South Africa (Table 2).

Figure 10: Map of the recent EQA-EMV 28, sent out January 2016 to 95 laborates in 30 countries with results from all countries, showing the hitherto widest distribution of the program.

Participants became involved also by personal contacts during national and international meetings on EM or by letters addressed directly to possible candidates explaining the conditions and advantages of the scheme. Several laboratories were highly enthusiastic initially, but finally did not enrol because of apparent "political reasons". Likewise, a few laboratories later dropped out because the EM lab was closed down for one or the other reasons, e.g., because of illness or retirement of the microscopist or the complete loss of commitment and interest in DEM within the institution.

Starting with EQA-EMV 9 up to EQA-EMV 18 much of the workload associated with the preparation of the EQA samples and the documentation was taken over by the technical assistant Andrea Männel (Fig. 11) and in part also by Lars Möller, then a student in biology. When Andrea Männel left the lab in 2005, Lars Möller took over these duties completely until this day. When Hans Gelderblom had to retire in due course in May 2004, Norbert Bannert became head of ZBS4 and changed the program from initially two distributions per year to only one (Table 1). From April 2011 on, the ZBS4 group (Advanced Light and Electron Microscopy) and the Consultant Laboratory are led by Michael Laue and he is assisted in the EQA-EMV program by biologist Lars Möller. The latest distribution EQA-EMV 28 was sent out by January 2016.

Table 2. Frequency of participation according to countries (from 1994 to 2016, EQA 1–28) The varying frequencies in participation are due in part also to a late recruitment.

country	frequency of participation	country	frequency of participation
Germany	28	Israel	15
Switzerland	25	China	14
Belgium	24	Lithuania	13
Italy	24	France	12
Spain	24	Malaysia	12
United Kingdom	24	Singapore	12
Czech Republic	23	South Africa	12
The Netherlands	23	India	11
Slovenia	23	Sri Lanka	11
USA	23	Kenya	8
Austria	22	Mexico	8
Japan	22	Argentina	7
Australia	21	Sweden	7
Canada	21	Norway	6
Finland	21	Greece	4
Russia	18	Cuba	1
Denmark	17	Portugal	1
Brasil	16	Romania	1
Ireland	16	Ukraine	1

Figure 11: Andrea Männel and Hans Gelderblom in the BSL-2 laboratory of ZBS 4 at Nordufer 20 at the laminar flow bench discussing EQA-EMV issues in 2004. Andrea took a major part in the EQA-EMV program between June 2000 and May 2005. Photo by Hans-Günter Bredow, RKI

The Role of External Reference Laboratories in the EQA-EMV Scheme

From the very begin of the EQA-program the quality of the test samples was controlled also externally. Presently they are evaluated by six independent reference laboratories. Based on their high DEM expertise these are selected by the Consultant Laboratory in DEM at the RKI from the group of participants in toto, but the selected laboratories are "blinded", i.e. not aware of their reference function. Only when 5/6 Reference Laboratories have diagnosed a specific specimen correctly, this sample is rated as applicable, i.e. of correct identity, free of other viruses, and sufficient in particle quality and concentration. This way, e.g., in EQA-EMV 17 (distributed November 2004), 2/6 virus suspensions were excluded from the final evaluation because more than one of the reference laboratories had failed in detecting these two agents.

Scope and Origin of the EQA-EMV Samples

As mentioned earlier the spectrum of the test agents was deliberately not restricted to either human or veterinary relevant pathogens because there is a wide overlap in their occurrence. There is a great number of important zoonoses, but we are also aware that some veterinary agents, e.g., birna- and iridoviruses, do not clinically infect humans. Nevertheless, we included those as well as recently the "new" mimivirus (Raoult et al., 2007) in the EQA-distribution as an opportunity to train the basic pattern recognition abilities. About half of the test samples were produced in-house, the others were supplied by colleagues from a great number of virus laboratories in Germany. Without this generous intra- and extramural support, this EQA-EMV program could not exist (see list of donors at the end).

How to Evaluate Success in EQA-EMV

Though participants were asked to handle the EQA specimens as routine DEM samples, it appears not unlikely that the samples were treated with special attention. They might have been prepared several times for DEM, by different investigators in the same laboratory and screened for prolonged periods of time. Therefore, the level of diagnostic success is probably artificially high as pointed out already by Reed et al. (1985 b). Nevertheless, also when treated with special attention, the EQA samples would have fulfilled their purpose as internal quality controls.

Taking into account inevitable differences in "quality", in, e.g., particle concentration and amount of "dirt" in the test specimens of the different EQA-EMV runs, we refrained from establishing a graded scoring system comparable to that of the PHLS's MQLC (Reed et al., 1985 a). The number of correct results of individual participants in the RKI scheme varied according to their expertise between 0 and 100 % per individual sample. Low performance rates, however, helped us to recognize individual laboratories with basic deficits in sample preparation and evaluation. Without disclosing "low performers", individual mistakes were discussed in the common "result letter". Occasionally also direct, personal advice was given on prints of the participant's micrographs (Fig. 7). This way, after 3–4 times attendance, the results also of newcomers in DEM reached accountable levels.

Lessons from the EQA

Rarely participants "detected" an agent in a negative control sample and likewise rarely participants reported the detection of two different viruses in an EQA suspension that clearly contained only one specific agent. False positives: how come? Cross-contaminations during specimen preparation in the DEM lab! Apparently, forceps and/or other tools used to handle the grids had not been cleaned during the preparation of different specimens - a cause for false positive results mentioned already by June D. Almeida (1983). Carelessness and clearly an unfortunate lapsus, but welcome to admonish adherence to proper methods in the next sample announcement and results letter.

And vice versa: probably due to a too rapid "prima vista-diagnosis", a few participants missed the second virus present in an EQA-EMV specimen that contained deliberately agents of two different virus families. Mixed suspensions were intended to train the participants in their stamina and endurance to detect double infections and in particular agents that might be present in lower concentration by adhering to defined rules for screening in DEM. I.e., a negative sample is considered "negative by DEM" only after scanning at least 20 "windows" on a well-stained 400 mesh grid or after 20 min overall of search.

Educational Measures Supporting DEM and the Aims of EQA-EMV

The EQA-activities are accompanied by a training program on DEM and related topics in biological EM consisting of Workshops, Lab-Meetings, and Basic Lab-Courses. Workshops are conducted annually either as satellite meetings in the frame of the conferences of the German Society for Electron Microscopy (Deutsche Gesellschaft für Elektronenmikroskopie, DGE), the first taking place in Leipzig in September 1995, or as autonomous 1.5 days conventions, the Glienicke-Meetings, accommodated initially in the Castle Glienicke, a quiet place in the West of Berlin, at the border to Potsdam. Workshops were attended by 40 – 80 participants, 90 % of them directly serving in DEM. Most of them came from Europe, including Russia, but regularly also from far distant countries like Canada, China, Japan, Kenya, Malaysia, USA, delegated usually from Public Health Institutions of their countries (see also Figs. 8 and 10 and Table 2). Participants from Medicine and Veterinary Medicine and representatives of commercial companies in the field of EM discussed here the role of DEM and teached and learned new technologies. Further Workshops were organized within the framework of international Microscopy or Virology Conferences, e.g., in Antwerp (2004), Bejing (2006), Bergen (1999), Brescia (2007), Brno (2000), Davos (2005), Hamburg (1998), Innsbruck (2001), Geelong and Kuala Lumpur (2004), Pune and Bangkok (2006), San Diego (2002), Sapporo (2006).

Basic Lab Courses: Requirements for Successful DEM

Beginners in DEM, and participants sending back meagre results were encouraged to attend special two-day courses at the RKI to improve their skills in preparation and evaluation. Here, main emphasis was put on (1) the preparation of suitably stable Pioloform-carbon specimen support grids, (2) how to achieve and test the adhesiveness (hydrophilicity) of the grids, (3) comparison of different NS contrast stains, (4) basic and reliable enrichment and immuno-EM procedures (Gelderblom and Reupke, 1978; Biel and Gelderblom, 1999 a), (5) deliberate labilization of particular stable viral envelopes by floating on distilled water and/or detergent, (6) measuring and counting using internal markers (Zheng et al., 1996; Luftig, 1968). Internal markers, like ferritin or gold-particles, catalase-crystals, adenovirus particles were recommended to admix to "critical" specimens before NS-DEM in order to control the required preparation performance. The courses were run twice a year with a restricted number of 9–12 participants, most of them from abroad. This way, about 200 participants were trained in 24 courses until now.

Arbeitskreis EM-Erregerdiagnostik (AK-EMED; Working Group on DEM of the DGE)

By September 10, 1999, on occasion of the Annual Meeting of the DGE in Dortmund, the AK-EMED was founded. It is headed by two speakers and consolidates DEM activities mainly in the German speaking countries. The Lab-Meetings of the AK-EMED are run annually, in German, and include also technicians and other staff members to avoid "difficulties" with the English language. Lab-Meetings are organized by individual, selected DEM laboratories at different places in Germany and represent the third institutional element of continuous education in DEM. They focus on lab techniques and exchange of practical knowledge. Besides educational lectures, they offer a fair chance to become acquainted with the peculiarities and methods of other DEM laboratories and thus improve networking. Recent and forthcoming activities - organized by Bärbel Hauröder, Koblenz - can be found on the website of the DGE (http://www.dge-homepage.de/akemed.html).

The Consultant Laboratory for DEM of Infectious Diseases at the Robert Koch Institute

Mid of 1995 the Public Health authorities together with the infectious diseases societies in Germany started to think about creating an effective infectious diseases network for the Federal Republic of Germany. Considering DEM an essential component of that "net" they appointed the existing DEM group at the RKI as the Consultant Lab for DEM of Pathogens (Konsiliarlabor für elektronenmikroskopische Diagnostik von Krankheitserregern) on June 23, 1997. The EQA-EMV scheme is registered and acknowledged also by the European Proficiency Testing Information System (EPTIS). Based on the lab's expertise in DEM and the continuing QC and educational activities, the appointment was renewed every two years also with the successors of hg, with Norbert Bannert (from June 2004 to December 2011) and Michael Laue (from May 2012 until today) by the Commission for Public Health Microbiology at the RKI (Wissenschaftlicher Beirat für Public Health Mikrobiologie beim RKI).

Relevance of the EQA in DEM

By September 10, 2001, at the DEM-Workshop held in Innsbruck during the Three-Country Meeting on Electron Microscopy, the role of rapid DEM in the diagnosis of outbreaks and biowarfare was discussed (Gelderblom, 2003 b). One day later, the 9/11 attacks shocked the world and only a few weeks later the bioterrorist anthrax letters in the USA caused five deaths from inhalational anthrax and >30.000 persons under surveillance and protective antibiotics (for review see c.f.: Jernigan et al., 2002). Based on the ease of production and spreading, the high morbidity and mortality of the disease and the ease of transmission and high panic potential, both, anthrax spores and variola major virus, the causative agent of smallpox, are considered likely bio-weapons (Lane et al., 2001; LeDuc and Jahrling, 2001; Meltzer et al., 2001). To raise preparedness and efficiency of DEM, a national telemicroscopy DEM network was established connecting the DEM-labs of the RKI and of six further DEM laboratories in Germany, initiated and headed by Josef Schröder, Regensburg (a joined proposal of hg and Josef Schröder, August 17, 2004). The IT system was demonstrated several times to work effectively. Considering the costs of this approach, however, it did not find support by decision makers.

Following 9/11, 2001 in the US, our lab distributed suspensions of different poxviruses in 10 % paraformaldehyde to train the respective skills and DEM abilities. Orthopox- and parapoxviruses (OPV, PPV) differ not only in size and overall shape, but also with regard to surface fine structure. PPV particles are smaller, ovoid (a bit "cigar-shaped") and surrounded by parallel running spirals. The larger, "brick-shaped" OPV, in contrast, are densely covered with short and randomly arranged envelope threats (for details see Fig. 12).

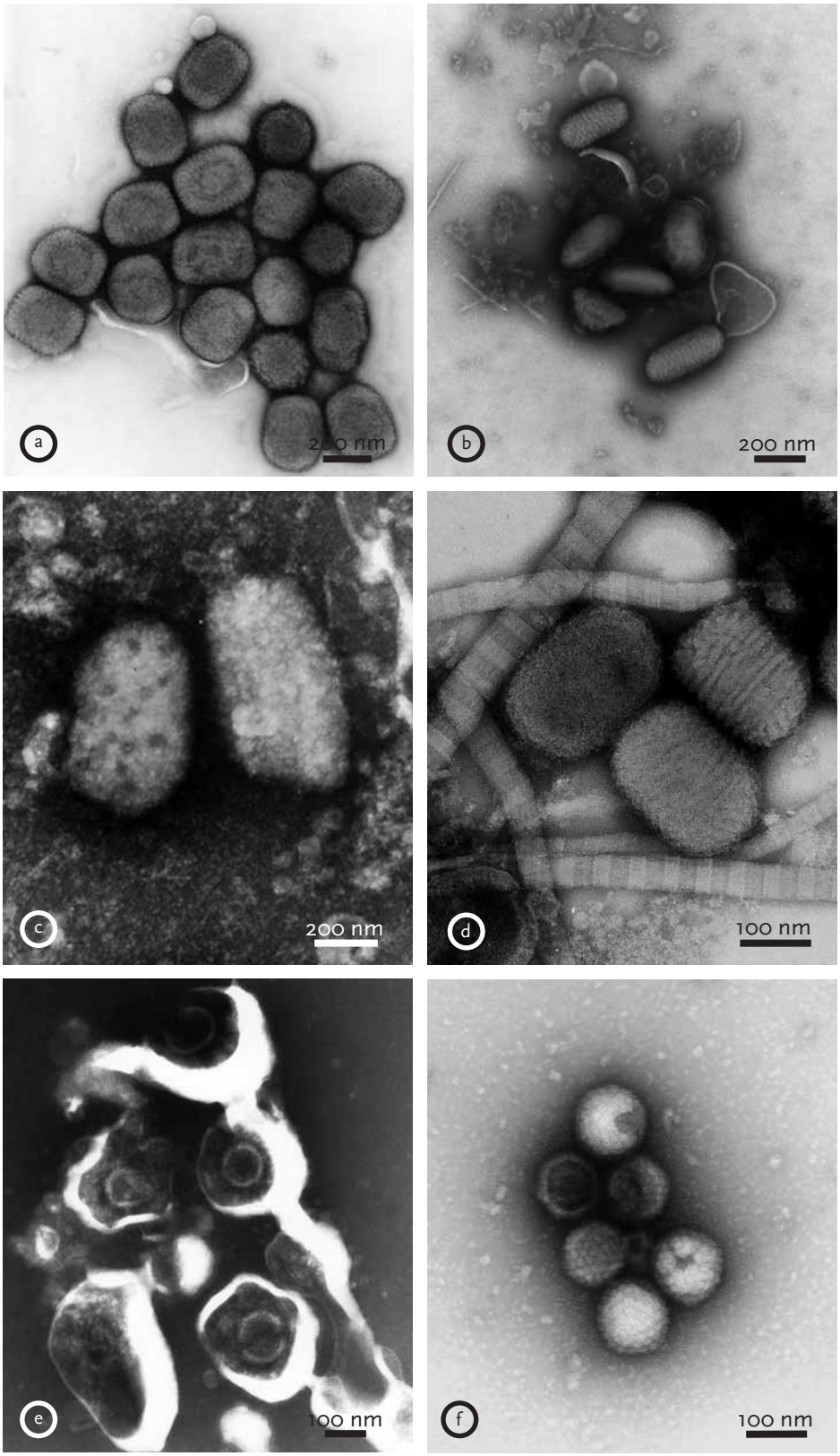


Figure 12: DEM of pox- and herpesviruses as well as of melanosomes after NS preparation.
(a) Ectromelia virus, a mouse-specific orthopoxvirus (OPV). Size (~400 x 220 x 150 nm), shows brick-shaped morphology and an irregular pattern of small surface differentiations, 15 nm in width and 30–40 nm in length, typical of members of the big OPV genus. It comprises the vaccinia virus and smallpox/variola virus (extinct in the field), camel-, cow-, monkeypox and further members (for more detail see c.f. ICTV, 2011). Photo by Andrea Männel, RKI

(b) Orf virus of sheep and other ungulates, the type species of the genus parapoxvirus (PPV). The sample originates from a fever associated human skin ulcer, caused by a zoonotic infection from sheep, and was propagated in cell culture. After Airfuge™ enrichment (Gelderblom and Reupke, 1978) and NS with 1 % PTA an abundance of ovoid particles 300 x 160 nm in size are shown with surface coils all around the virion, i.e. a typical parapoxvirus fine structure. Further members of the PPV genus are bovine pustular stomatitis virus and pseudocowpox virus (for more detail see c.f. ICTV, 2011). Photo by Andrea Männel, RKI

(c) Two melanosomes, i.e. pigment particles of melanocytes (specialized cells in the skin), set free when skin biopsies are grinded. When DEM specimens are not well "stained" the unexperienced may interpret such structures by size, up to 500 nm in length and ill-defined surface pattern, as poxviruses. Photo by hg, RKI

(d) Molluscipoxvirus (MOCV) from a human wart ("Dell-Warze") biopsy prepared after grinding as in (b). MOCV is transmitted by direct contact or in direct, smear contact often between children, and causes opportunistic infections in immuno-compromized persons. Specialists in DEM can morphologically differentiate MOCV by surface structure and overall shape from the similar OPV. The virions are catched here in a network of collagen fibers, a physiological component of connective tissue with its typical periodicity of 64 nm. Photo by hg, RKI

(e) Varicella-zoster herpes virus (VZV), i.e. human herpesvirus 3 (HHV-3), the cause of chickenpox and herpes zoster, was the most likely alternative in DEM in the differential diagnosis of smallpox. Both, variola major and VZV, cause febrile vesicular rash diseases. In adults, the VZV infection often resembles smallpox, and vice versa, a mild smallpox infection, especially in children, can closely resemble chickenpox. The causative agents, belonging to the pox- and the herpesvirus families respectively, are differentiated rapidly by direct touch DEM as the lesions contain ample particle concentrations of $>10^8$/ml. By NS-TEM all herpesviruses resemble each other. However, TS-TEM can reveal morphological differences, e.g. in the expression of the tegument layer. The complete virion is enveloped and measures 150 to 180 nm in diameter. Occasionally surface projections 12–15 nm in length are seen, not detectable here in the relatively "deep" PTA. It hides under a tegument layer an isometric core-capsid of 100–110 nm. The viral envelopes and the cores are broken due to osmotic damage and the shrinkage of the drying PTA-stain. Herpesvirus and adenovirus capsids are icosahedral, but differ in size and capsomer numbers. The herpesvirus capsid is composed of 162 capsomers, the ~85 nm adenovirus capsid of 252 capsomers. Photo by hg, RKI

(f) Bovine herpes virus type 1 was propagated in cell culture. During gradient purification, the virions lost their envelopes, presenting a clean fraction of naked isometric nucleo-capsids. Three of the capsids are partly penetrated by the electron-dense UAc stain, while the remaining three capsids reveal a faint 162 capsomere pattern. Photo by Andrea Männel, RKI

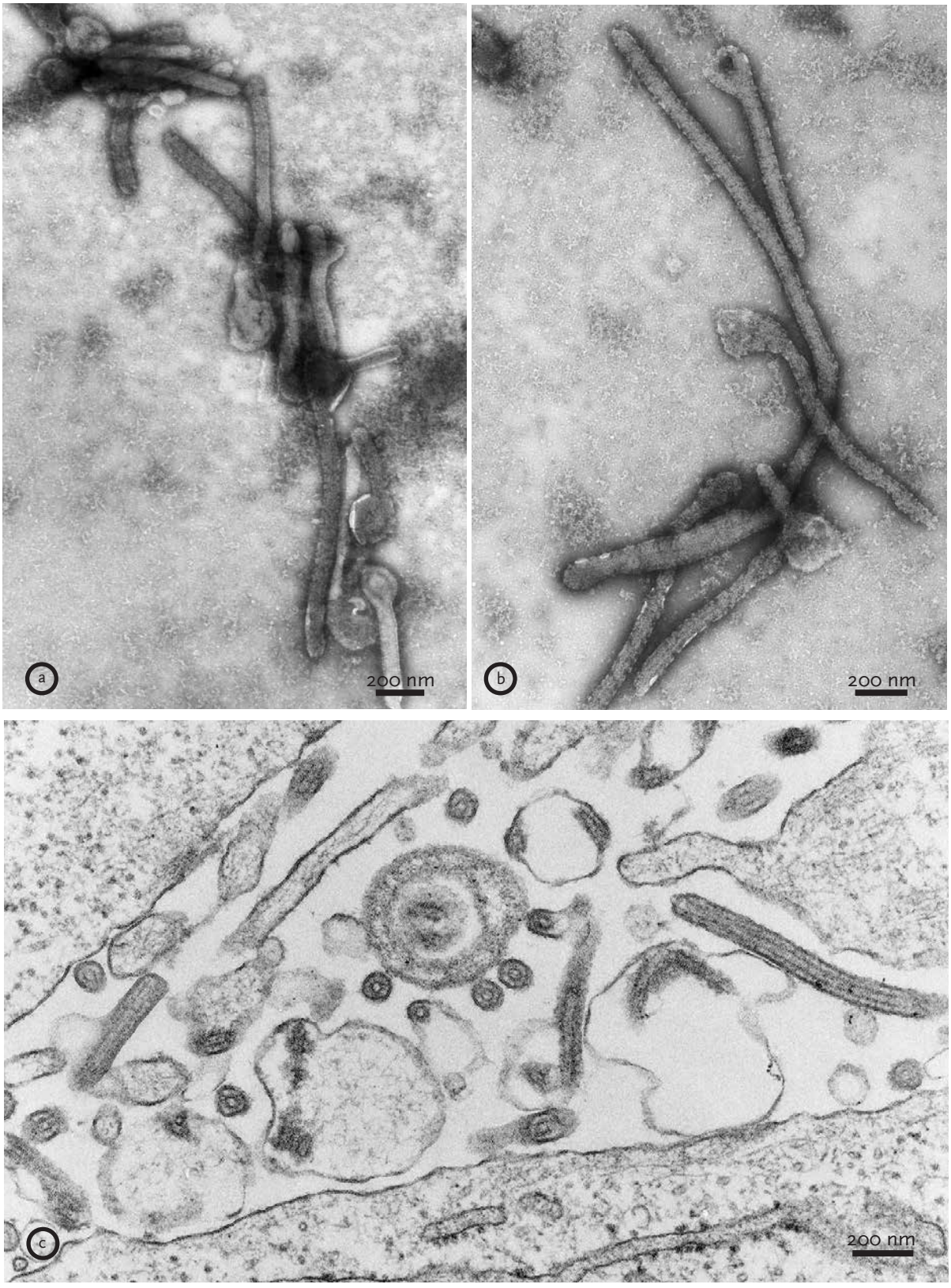


Figure 13: A comparison of Marburgvirus (a) versus Ebolavirus (b). Purified and inactivated viruses were obtained in 1980 from Werner Slenczka, Marburg. Both viruses are filamentous, hence the designation of the virus family *Filoviridae*. The agents have a diameter of 80 nm and contain an inner ribonuclear protein helix 50 nm in diameter. They differ, however, distinctly in genetic and antigenic make-up, in length and in vitro by the formation of different inclusion bodies (Geisbert and Jahrling, 1995; Goldsmith, 2014; Peters et al., 1971; Ryabchikova and Price, 2004). Marburgviruses measure about 800 nm in length, while Ebolaviruses are generally longer, often exceeding with great variation the length of 2 to 5 micrometer. NS both with 2 % PTA. Photos by hg, RKI

(c) Marburgvirus release by budding from the surface of a Vero cell. Depending on the orientation in the section, longer viral filaments are revealed rarely, more often transverse cross-sections are seen. Inside the "filaments", the viral ribonucleoprotein with a diameter of 50 nm is clearly evident. Photo by hg, RKI

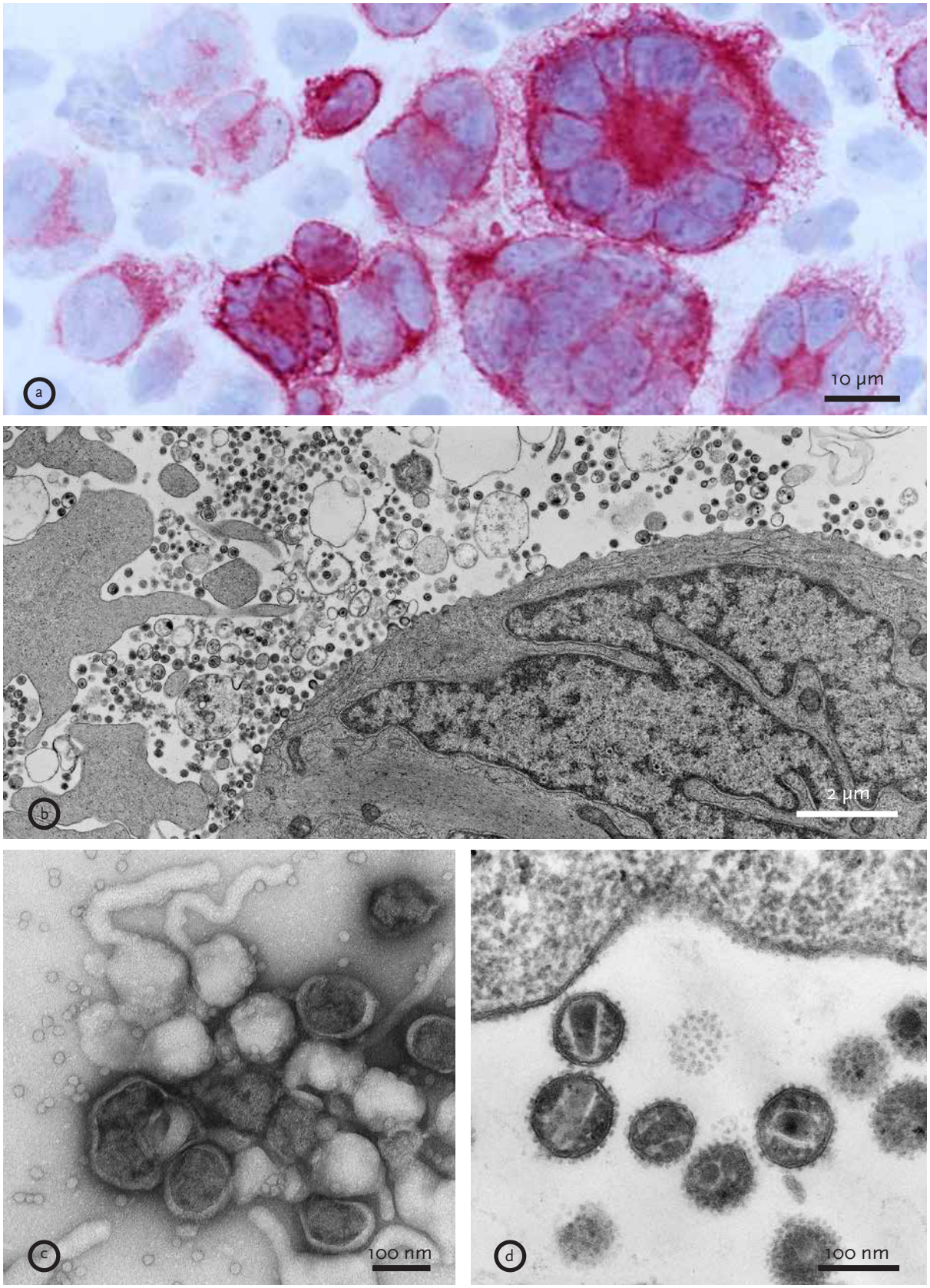


Figure 14: Work on human and simian immunodeficiency viruses (HIV, SIV; Gelderblom and Boller, 2002):
(a) Formation of multi-nucleated syncytia by fusion of HIV-1 producing H9-cells with un-infected H9-cells. Immunocytochemistry using specific antibodies and alkaline phosphatase/anti-alkaline phosphatase (APAAP) labelling reveals viral p24 core antigen (red) in the cytoplasm of multi-nucleated cells. Nuclei are counterstained in blue, while uninfected cells are weakly blue. Specimen courtesy by Rudolf Kunze. Photo by hg, RKI

(b) TS-TEM of a multi-nucleated cell showing an abundance of budding and cell-released SIV agm (african green monkey) particles. Photo by hg, RKI

(c) HIV-2 was propagated in H9-cells and purified by Percoll-gradient ultracentrifugation. After NS with 1 % PTA the virion fraction reveals a high degree of

structural lability. Many virions are penetrated by the stain, while others show tail-like protrusions, i.e. extensions of their labile envelopes due to osmotic damage. The fraction was kindly provided by Professor Karl - Otto Habermehl, FU Berlin. Photo by hg, RKI

(d) TS-TEM of simian immunodeficiency virus (SIV) SMLV STLV-III agm in MT2 cells revealing different section planes- 50–60 nm in thickness - through the virions, which themselves measure 120 nm in diameter. The viral core in these and other morphologically mature lentiviruses is cone-shaped and associated on its long axis by dense lateral bodies (Gelderblom et al., 1987 a). The viral envelopes are more or less densely studded with viral surface projections, indicating a time and temperature dependent shedding of viral glycoprotein. The round crowd of faintly stained tri-angles in the upper middle represents the uppermost portion of the virion, contained in a tangential section plane. We interpreted the tri-angles and their density early on as an indication for the trimer structure of the lentiviral glycoprotein, a decade before finally molecular biology detected and believed the reality of trimers (Gelderblom et al., 1987 a; Gelderblom and Boller, 2002; Özel et al., 1988). Photo by Freya Kaulbars, RKI

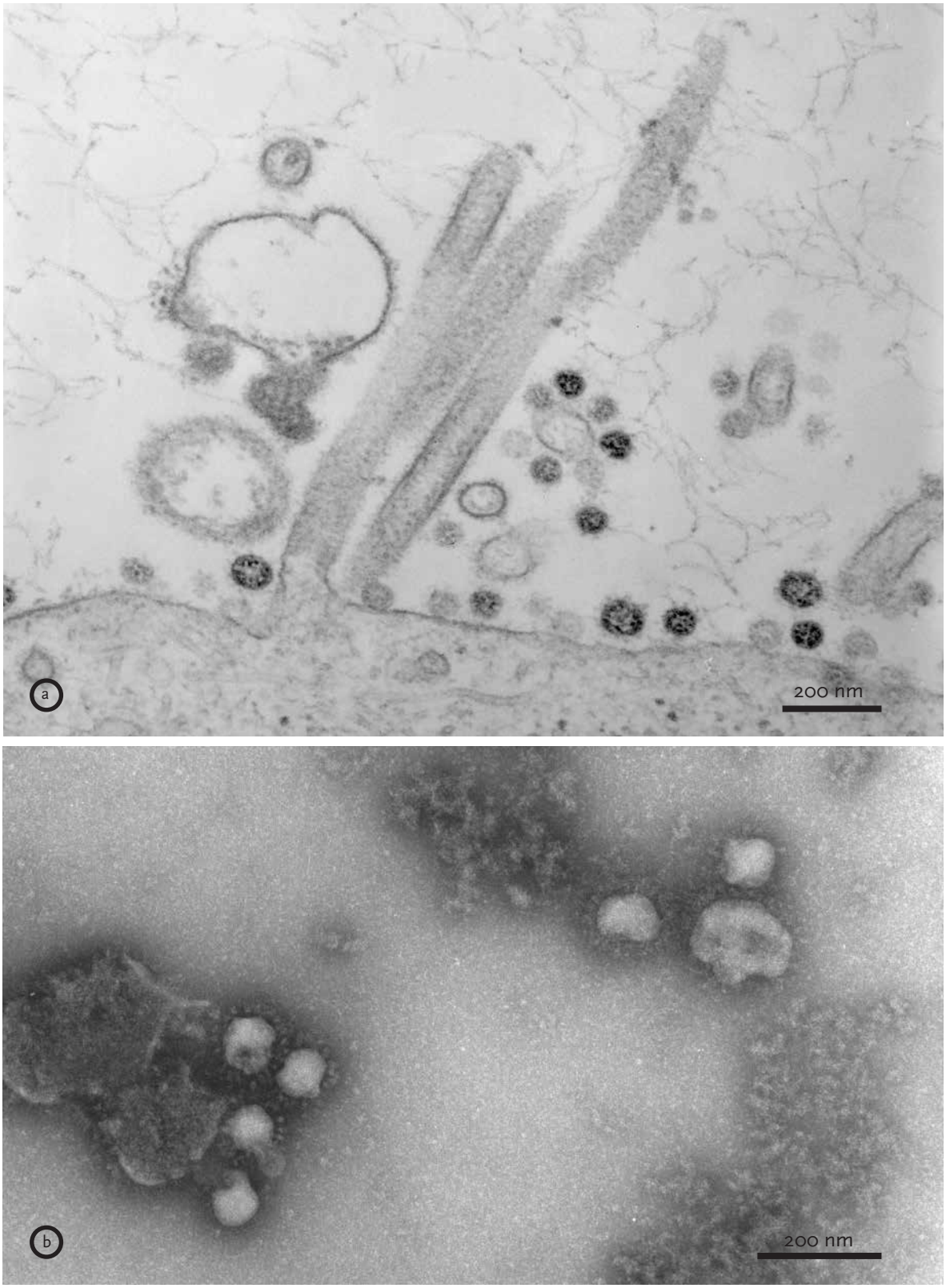


Figure 15: SARS-coronavirus by TS-EM (a) and NS-TEM (b).
(a) Same-day TS-TEM of SARS-coronavirus Frankfurt infected Vero E6 cells. Mature, cell released coronavirus is seen as dense, round enveloped particles 120–140 nm in size. They are studded faintly with an incomplete fringe of surface glycoprotein, club-shaped peplomers 20 nm in length. From the same cell body, also paramyxovirus (PMV) is budding, a contamination in many permanent cell cultures. The PMV RNP is seen in cross-section as tiny, 20 nm rings underneath the stiffened lipid bilayer. The microwave assisted rapid embedding was performed by Josef Schröder, Regensburg (2006). Photo by Freya Kaulbars, RKI

(b) NS-TEM of SARS virus from the supernatant of a Vero cell culture, fixed with 2 % formaldehyde, sedimented using the Airfuge onto carbon-reinforced Pioloform copper grids and stained using PTA. Despite the culture derived detritus the peplomers - typical of members of the Coronaviridae - are seen clearly on the viral envelopes. Photo by Andrea Männel, RKI

When skin biopsies are grinded and suspensions studied by NS-TEM, besides collagen fibres (Fig. 12d) not too seldom also melanosomes (Fig. 12c) can be detected. The latter can mimic OPV (Fig. 12a) by shape and size. Therefore, a safe diagnosis "OPV particles" requires both, measurements of particle dimensions and a clear and distinct presentation of the viral surface. Overall, the technical and diagnostic expertise of the participants in EQA-EMV attained respectable levels. When the Consultant Lab was asked by not participating microscopists to have a look at "suspicious" findings, we learned repeatedly that participants in EQA-EMV outperformed other, definitely esteemed life science EM laboratories not only in diagnostic experience, but remarkably also in the quality of NS preparation. Indeed, over the last decade a deplorable loss of quality in routine life science TEM is noted (Ackermann, 2014). Therefore, to improve preparation techniques was another intention when starting EQA-EMV at RKI.

Highlights in DEM and Research until 2004

Our in-house activities focused mainly on dangerous or lethal pathogens. The fine structure of Ebola- and Marburg (filo) viruses was studied in 1980 by comparative TS- and NS-TEM in collaboration with Werner Slenczka, Marburg (Fig. 13). EM turned out a leading edge technique in HIV research (Gelderblom et al., 1985, 1987 a, b; Gelderblom and Boller, 2002; Grewe et al., 1990; Özel et al., 1988) generating much interest (Fig. 14). Different hantaviruses were studied between 1998 – 2004 in collaboration with Detlev H. Krüger, Berlin (Geldmacher et al., 2004), herpesvirus B with Hanns Ludwig, Berlin (Ludwig et al., 1983), iridoviruses with Gholamreza Darai, Heidelberg (Jakob et al., 2002), paramyxovirus with Christian Grund, FLI Riems (Grund et al. 2002), and SARS-coronavirus (Fig. 15) with Hans W. Doerr, Frankfurt and Jan ter Meulen, CRUCELL Leiden, NL (ter Meulen et al., 2004). Pathogenic bacteria were studied during the years 2001 – 2004 with the enthusiastic support of Rolf Reissbrodt from the Bacteriology Department of RKI Wernigerode (Willems et al., 2002). It was in particular this wide spectrum of agents and collaborations - besides the comprehensive in-house virology from adenoviruses to HIV and scrapie - that helped to acquire the expertise that was asked for by the Ministry of Health, e.g., when monkeypox virus or when in 1989 the Reston-Ebolavirus turned up in Indonesia, the US, and in Germany. Despite the lack of a profound, serious pathogenicity in humans of the latter two agents, our humble opinion proposed to establish a High Security Laboratory aside the RKI to take care of future worst case outbreaks caused by emerging or re-emerging pathogens. In 2018, the BSL 4 Laboratory of the RKI at Seestrasse will go in full operation. To the success and the comprehensive knowledge of the group contributed the basically favourable research conditions at RKI yet also a number of long term visiting scientists (Volker Brinkmann, Berlin; Stefan Höglund, Uppsala; Massimo Gentile, Rome; Xiaolin Zhang, Bejing; Jörg-Peter Rabanus, San Francisco) and the long series of dedicated students by their diploma or thesis work. The achievements were acknowledged by the AIDS-Forschungspreis of the Deutsche Gesellschaft für Infektiologie in 1988 and the Loeffler-Frosch Medal of the Gesellschaft für Virologie in 2013 to hg. After 9/11 and the anthrax letters in the US, Germany and many further countries were hit by waves of suspected bioterrorism and also our group became engaged frontline. The first serious BT suspects in Germany, two supposed anthrax isolates, came by helicopter transport to Berlin the evening of November 02, 2001. NS-DEM revealed faint fimbriae with the bacterial body and made a diagnosis "B. anthracis" very unlikely as genuine anthrax bacilli do not express fimbriae or pili (Fig. 16). Accordingly, investigations by the Bacteriology Group of Centre for Biological Threats and Special Pathogens (ZBS) arrived at the diagnosis B. megaterium, the next day (Daniela Jacob, RKI, 2014, personal communication).

On the Role of DEM at the Robert Koch Institute

At RKI, NS-DEM is used as an indispensable part of the diagnostic repertoire in cases of presumed bioterrorism, in parallel to NADT, cultivation, TS-TEM, and spectroscopy/spectrometry (Gelderblom et al., 1991; Gelderblom, 2003 a; Gentile and Gelderblom, 2014; Hazelton and Gelderblom, 2003; Kull et al., 2010, Kurth and Nitsche, 2007; Lasch et al., 2009, Laue and Fulda, 2013; Nitsche et al., 2006). The analysis revealed all kinds of mock and natural environmental or household substances.

Technical improvements were introduced, which help in practice to analyse such samples. NS preparation variables for poxviruses and bacterial endospores have been analyzed and a defined detection limit was established for NS-DEM (Laue and Bannert, 2010). The detection of spores in environmental samples has been optimized by using single-particle X-ray spectroscopy in conjunction with NS-DEM (Laue and Fulda, 2013). Likewise, rapid and reliable rapid embedding techniques for TS-TEM were improved (Laue et al., 2007).

DEM supports medical diagnosis in outbreaks of severe infectious diseases [e.g., the huge gastroenteritis outbreak in 2012, which affected over 10.000 children in Eastern Germany (Höhne, 2012)], and in individual cases of diseases with unknown etiology. DEM can quickly provide a decisive differential diagnosis, or a tentative diagnosis for a disease causing pathogen and can control the results established with other methods. Due to the inherent speed and open view a morphology-based diagnosis offers essential advantages in emergency cases where quick and reliable information is needed because otherwise consequences would be dramatic (e.g., public panic, false medical treatment).

NS is a question of minutes and is by far the quickest method in DEM and therefore the method of choice in emergencies. However, the simple NS-DEM is confined merely to the family diagnosis of a detected agent. For a diagnosis down to the species level, for virus typing, a couple of sensitive immuno-EM techniques can be applied (Biel and Gelderblom, 1999 a; Gelmetti et al., 1996; Lavazza et al., 2015; Miller and Howell, 1997). TS-TEM is used when information on the cell tropism of a pathogen or on aspects of its replication and/or release is required. Several rapid protocols provide a result within a few hours (Schroeder et al., 2006; Laue et al., 2007; Zechmann et al., 2011). An example for the application of rapid microwave assisted TS-TEM (Schroeder et al., 2006) was the characterization of several SARS coronavirus isolates (Fig. 15).

Diagnostic cell cultures, inoculated with suspected clinical material, are routinely analyzed merely by NS-DEM. Culture supernatants are investigated directly or after enrichment or pellets of supernatants after freezing and thawing of the cultures (Biel and Gelderblom, 1999 a; Goldsmith and Miller, 2009; Goldsmith et al., 2013). However, TS-TEM of the infected cells or of ultracentrifuge pellets of respective supernatants allows a more reliable DEM and the exclusion of common contaminations like mycoplasma, or reo- and paramyxoviruses (see Fig. 15 a). It also helps in characterizing details of the virus-cell interaction and to correlate cytopathic effects observed by light microscopy with the ultrastructure of the cells (Fig. 14).

Perspectives of EQA in DEM

Considering the role of DEM in emergencies and the overall decrease of samples for DEM, preservation of skills and hence diagnostic quality is an ongoing task. Different from the modern molecular techniques, DEM requires specific talents as stated recently: "Some people are inherently 'visual' and others 'auditory' and the former might make good microscopists but the latter probably never would, even with careful training" (Dick Madeley, Newcastle, UK, 2014, personal communication). Success in DEM is based on good laboratory techniques, yet it depends also on "weak factors" that are less decisive with ELISA and NADT techniques. The latter can be automated and controlled to a high degree, but in DEM a lot of positives will be missed without robust expertise and motivation (see also: Wolfe et al., 2005).

How to learn DEM?

It is like boxing! You cannot learn it from a book. Textbooks and virus atlases will teach you a basic, though only "theoretic" knowledge. Only the own engagement with the educational EQA-EMV samples and a certain in-house sample throughput in DEM will generate a robust expertise, visual imprints and a sound understanding of the 27 different virus structures found within the 27 mammalian specific virus families (ICTV, 2011). To narrow the intellectual and diagnostic gap between well established viruses and their morphologies and any hitherto undescribed "new" virus the EQA-probes comprise also agents not observed in human virology, e.g., birna- and iridoviruses in EQA-EMV 10. Recently, in EQA-EMV 24, also a non-vertebrate agent, the mimivirus, an in many aspects unconventional agent (Raoult et al., 2007) was presented in order to train imagination and pattern recognition abilities. The same purpose served the "dia-gnostic quiz", a 20 min slide-discussion, usually at the end of a DEM workshop or course. To be aware of a wide spectrum of morphologies in DEM appears essential as pointed out also by Wolfe et al. (2005): "rare items often missed in visual searches". I.e. only a widely and well trained microscopist will detect "new" structures with some certainty. Participants with robust pattern recognition abilities will benefit also from a regular, high diagnostic sample throughput. In this way, DEM is able "for identifying new pathogens" also in bacteriology (Fig. 16) and parasitology (Curry, 2000). Participation in EQA-EMV is raising the diagnostic safeness, speed and self assurance and thus enables to diagnose also incomplete virus structures and virions undergoing deformation or decay. The high level of technical performance of the participants in EQA-EMV is shown in Fig. 17.

Perspectives of DEM in Infectious Diseases

During the last decades we experienced the emergence and re-emergence of a great number of dangerous infections. Our life style, i.e. global traffic, war, refugees, and industrial crop and meat production are among the key factors fostering emergence and spread of „new" pathogens (Morens et al., 2004). The control of communicable diseases depends primarily on reliable lab diagnostics. Here the speed and open view of DEM can play its unique role either by a rapid differential diagnosis or supporting other lab diagnostic methods. DEM is visualizing also pathogens the physician did not think of before and also pathogens produced by synthetic biology (Petro et al., 2003; Wimmer et al., 2009). Such pathogens could combine several known but also hitherto unknown features. E.g., a mismatch or contradictory match of the diagnosed features with features of known pathogens could prevent a proper diagnosis even by specialized lab diagnostics. In such difficult cases, a wider range of techniques including diagnostic cultures and DEM should be used (Nolte et al., 2004) for correlating the genetic information obtained with the „new" properties of the underlying infectious pathogen. Because of the intrinsic capability to visualize any „nanoparticle" present in the sample down to the practical resolution limit (of at least 2 nm) DEM can play a key role in the detection of synthetically modified pathogens. In detecting also the un-expected, DEM provides a first description of the suspicious particle that helps to guide further search and identification strategies.

Routine diagnostics of infectious diseases is increasingly done by automated molecular techniques such as immunological tests and NADTs. Nevertheless, the generic method of DEM with its „open view", which is able to detect any pathogen in a sample, will be indispensable especially in cases where time is critical and reliability of the diagnosis must be high (e.g., outbreaks, cases of presumed bioterrorism, life-threatening infections) to prevent spread of infection or to treat patients quickly. DEM may lead to a diagnosis in conjunction with clinical data, or may guide the search and the specific identification of a pathogen by providing specific hypothesis for the presence of a pathogen, which facilitates the choice of specific probes or narrows down the range of data to be analyzed. Yet even when the most recent NATDs, like NGS (next generation sequencing, which is generic by definition), improve further in speed due to improved bioinformatics, the open view of DEM will be used to prove the presence of intact agents in a sample - structural integrity being an indication of infectivity and pathogenicity.

For the sake of an unbiased presentation of DEM, also first steps to an „automatic virus identification using TEM" should be mentioned here (Kylberg, 2014; Ong and Chandran, 2005; Utagawa et al., 2002). These attempts are based on the recent tremendous progress in IT-techniques and computer capacities. However, they appear still too slow and do not cover the entire spectrum of viruses. From the above, it becomes clear that application of DEM is relevant in emergency cases, which, though rather low in number, can have tremendous impact on individual or public health. It is therefore necessary to conserve capabilities of DEM for Public Health preparedness. To make optimal use of DEM, it needs to be applied frontline, and it must not be confined to just one or two centers of competence per country. DEM should remain in wider use also at universities, public health institutions and research organisations. To guarantee its high performance, DEM should be run integrated in the framework of standard life science EM (Biel and Madeley, 2001; Gentile and Gelderblom, 2014). Both, EM in basic science and DEM, will benefit mutually, a strategy followed at RKI with some success (Fig. 18). The synergy of EQA-EMV, of workshops and lab courses provides continuous education in DEM to keep the standards high and facilitate its robust future (Curry et al., 2006; De Haro and Furness, 2012; Gelderblom, 2001; Gentile and Gelderblom, 2014).

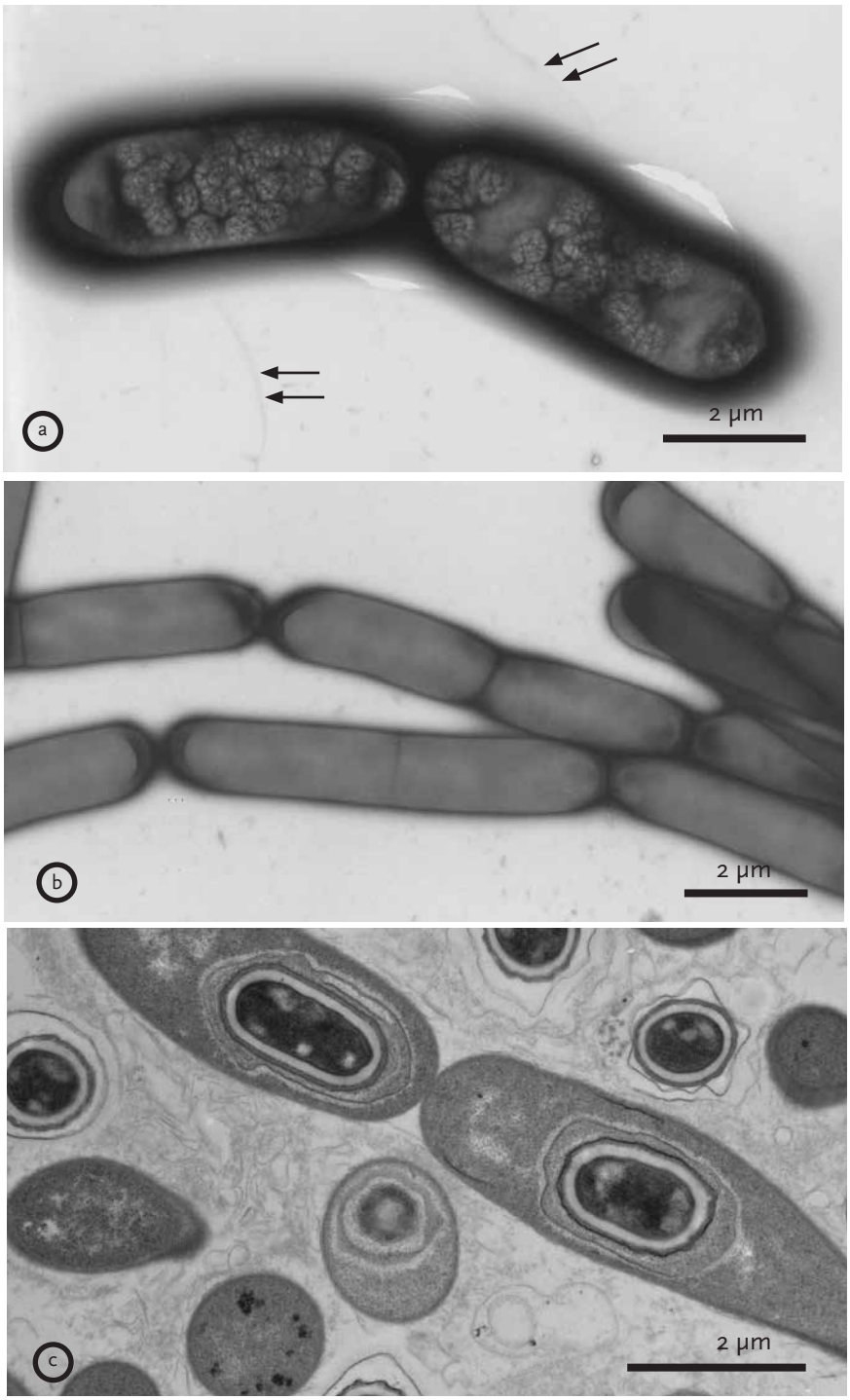


Figure 16: (a) A bioterrorism (BT) suspected „anthrax" isolate, flown on November 02, 2001 by heli to Berlin was received 05:00 p.m. in the EM lab. Thirty minutes later, rod-shaped bacteria were seen in chains and possessing faint fimbriae (arrows). The small „clouds" inside the bacterial body are „UAc-staining artifacts". As the presence of fimbriae is not compatible with common B. anthracis, a case of BT was ruled out. The next day, the Bacteriology Department identified the isolate as Bacillus megaterium. NS with 1 % UAc. Photo by Muhsin Özel, RKI

(b) A positive control sample of Bacillus anthracis, obtained from the Bernhard-Nocht-Institut in Hamburg showed the typical growth in chains, however, was completely devoid of, as expected, any fimbriae or flagella. Negative staining with 0.5 % UAc. Photo by hg, RKI

(c) TS-TEM of B. anthracis after rapid embedding, kindly performed by Josef Schröder, Regensburg (2006) showing free and still cell-associated spores. Photo by Lars Möller, RKI

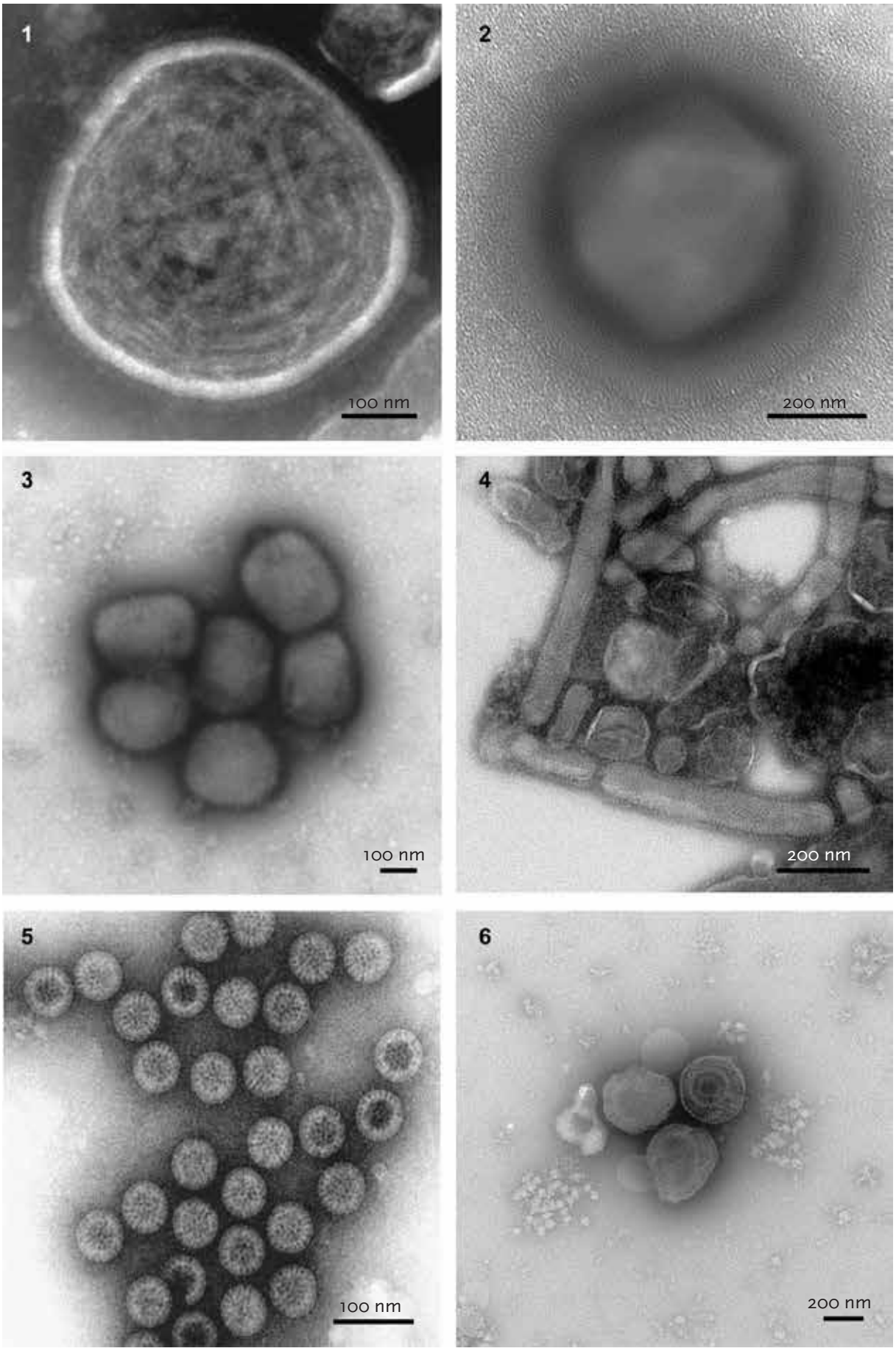


Figure 17: A gallery of DEM-results of the participants in EQA-EMV 26: brightness and contrast were adjusted and images were labeled with inscriptions. 1: Sendai virus, Antonie Neubauer-Juric, Germany. 2: Acanthamoeba polyphaga mimivirus, Oleg Taranov, Russia. 3: Vaccinia virus, Lynn Burton, Canada. 4: Influenza A virus A/H3N2, Silke Loch, Germany. 5: Human rotavirus A, A. S. Santhana Raj, Malaysia. 6: Human cytomegalovirus, Maureen Metcalfe, USA.

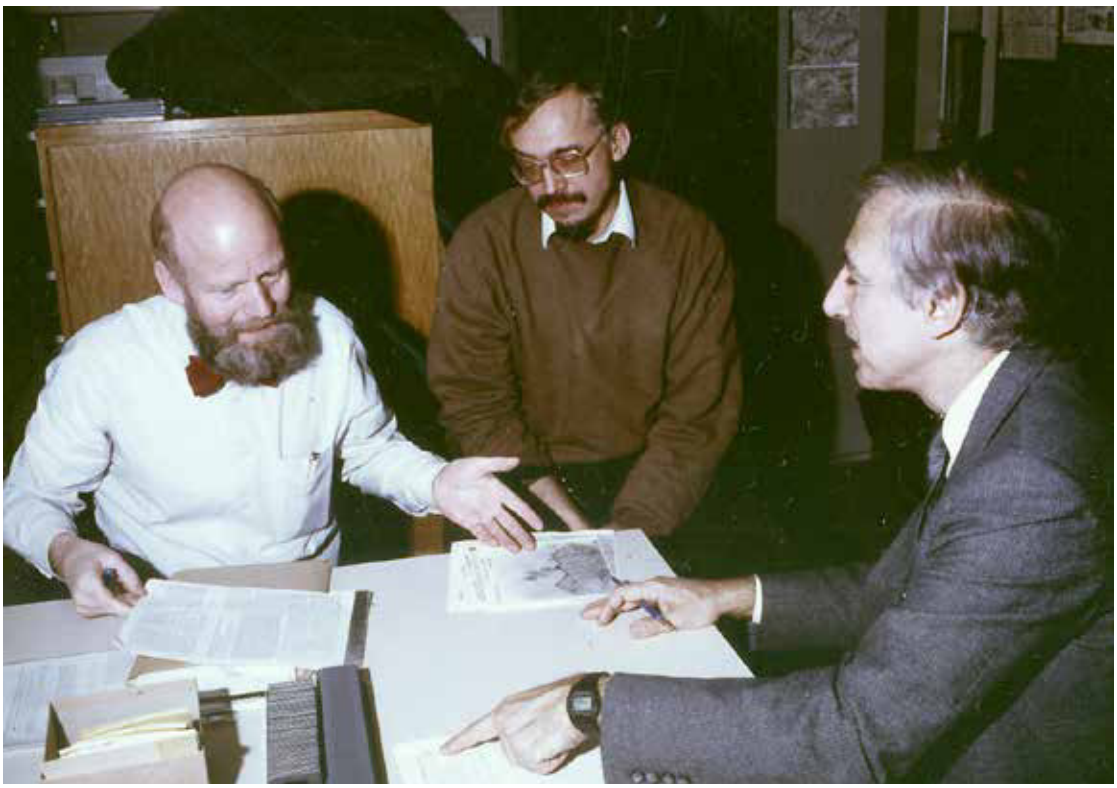


Figure 18: (a) The achievements of the EM-group caused some interest - we enjoyed many visitors from science and from Public Health. In 1987, Bob Gallo, Georg Pauli and hg are discussing recent results on HIV and SIV. Gallo had generously provided us with HIV-1, susceptible cells and antibodies enabling studies, on cell biology, epidemiology and immunology of HIV as well as leading edge structure-functional EM (Gelderblom et al., 1985, 1987 a b; Özel et al., 1988) by Georg Pauli, hg and their colleagues (see also Fig. 14). Meinrad A. Koch and President Großklaus then generously supported our efforts.

(b) The Secretary of Health and Human Services Andrea Fischer at the RKI on April 30, 1999. Discussion of Scrapie-associated fibers in the context of "mad cow disease" at the Zeiss EM 10 TEM, a most reliable "working horse". The EM 10 purchased in 1979 as the second TEM in our group "produced" about 79.000 negatives and was replaced by a fully digitized FEI BioTwin in 2005. The EM 10 was preferred also as a student's training instrument and after its life at RKI is operated now by Freya Kaulbars at the Lise Meitner Technical High School in Berlin. We were satisfied with the speed, the procedures and the results of the wet lab era. The workflow was fast: images recorded in the evening were available the next day in the early morning for further processing because the photo technician Barbara Jungnickl (January 1982–June 2012 at RKI) used to start work at 5 a.m. - a perpetual point of discussion with RKI's administration. Freya Kaulbars to the right, the Secretary sitting, hg to the left. Photo by Andrea Schnartendorff, RKI

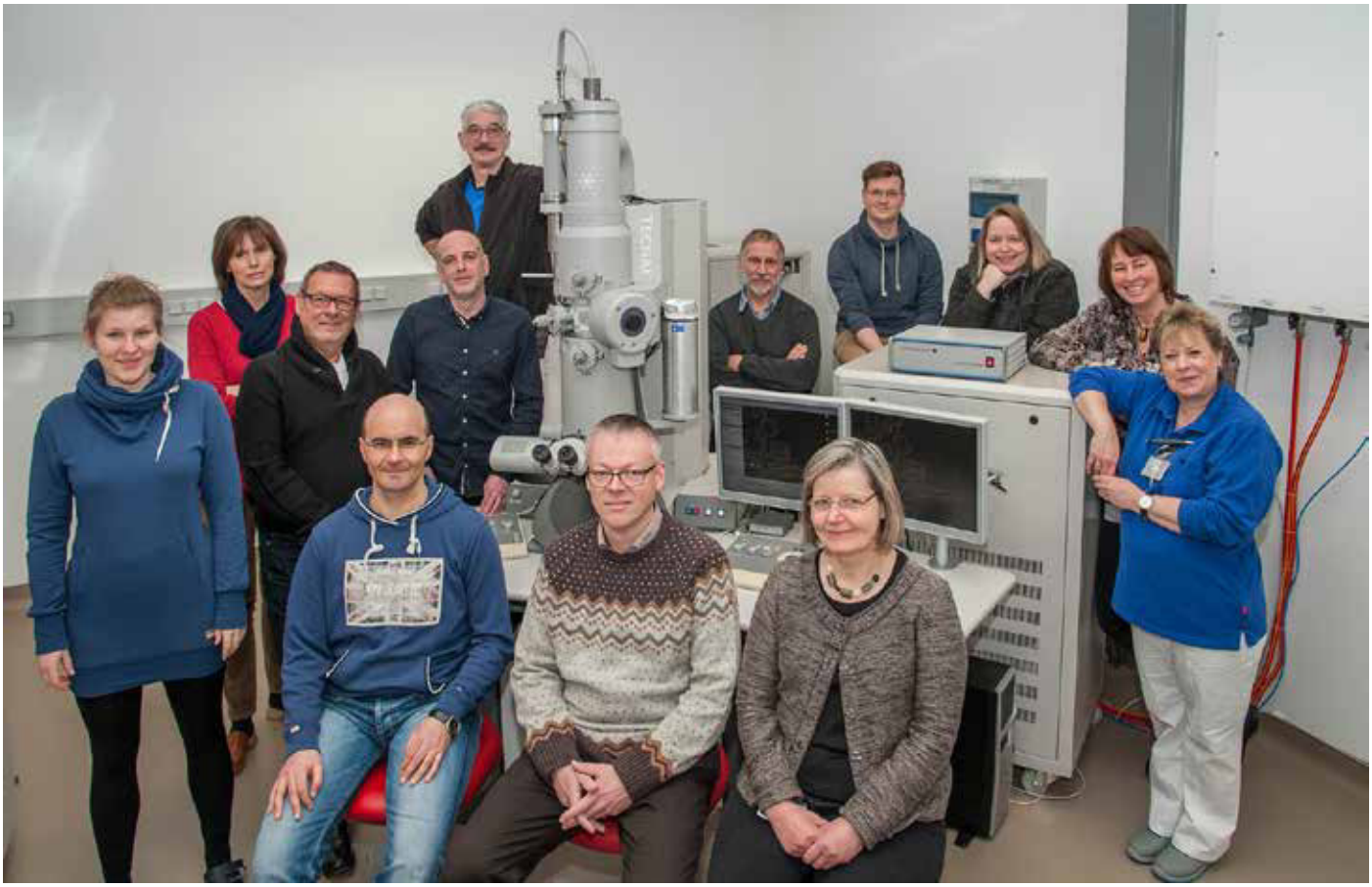


Figure 19: Working group Advanced Light and Electron Microscopy (ZBS4) in 2016, (left to right), Christin Dittmann, Petra Kaiser, Hans-Günter Bredow, Christoph Schaudinn, Lars Möller, Gerold Ruf, Michael Laue, Kazimierz Madela, Gudrun Holland, Marc Schumann, Janett Piesker, Andrea Schnartendorff, Petra Baer.

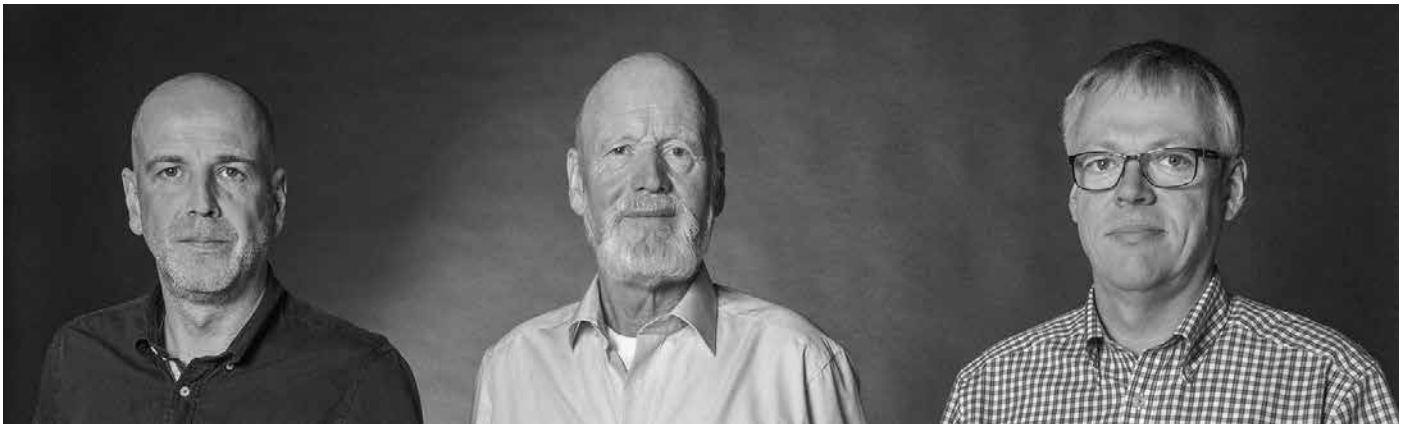


Figure 20: The authors (left to right), Lars Möller, Hans R. Gelderblom and Michael Laue in August 2016. Photo by Hans-Günter Bredow, RKI

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List of abbreviations

EM	=	electron microscopy
EQA-EMV	=	External Quality Assurance Scheme in Electron Microscopy of Viruses
hg	=	Hans Gelderblom
NADT	=	nucleic acid based detection techniques (i.e. PCR, next generation sequencing)
NS	=	negative staining, preferred technique to prepare suspensions of particles for TEM
QC	=	quality control
RKI	=	Robert Koch Institute, Berlin
TEM	=	transmission electron microscopy
TS-TEM	=	thin section TEM

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