REVIEW

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The Role of Electron Microscopy in the Rapid Diagnosis of Viral Infections – *review*

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ABSTRACT. Electron microscopy (EM) allows fast visualization of viruses in a wide range of clinical specimens. Viruses are grouped into families based on their morphology. Viruses from various families look distinctly and these morphological variances are the basis for identification of viruses by EM. The identification to the family level is often sufficient for the clinician or recognition of an unknown infectious agent. Diagnostic EM has two advantages over enzyme-linked immunosorbent assay and nucleic acid amplification tests. After a simple and fast negative staining, EM allows fast morphological identification and differential diagnosis of infectious agents contained in the specimen without the need for special considerations and/or reagents. Nevertheless, EM has the disadvantage of being unsuitable as a screening method.

Abbreviations

CSF	cerebrospinal fluid	PBS	phosphate-buffered saline
ELISA	enzyme-linked immunosorbent assay	PCR	polymerase chain reaction
EM	electron microscopy	PTA	phosphotungstic acid
IEM	immune electron microscopy	SARS	severe acute respiratory syndrome
NAT	nucleic acid amplification (techniques)	SPIEM	solid phase immune electron microscopy

NRL/ELM *National Reference Laboratory for Direct Diagnosis of Viruses, Borreliae and Foreign Cell in Clinical Specimens*

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1 INTRODUCTION

In the early 1960s, electron microscopy (EM) became widely used in viral diagnosis, primarily thanks to the introduction of the negative staining techniques (Brenner and Horne 1959; Hayat and Miller 1990; Harris 1997). During the 1970s and 1980s, EM made it possible to detect in diagnostic cell cultures many clinically important infectious agents such as adenoviruses, enteroviruses, orthomyxoviruses, paramyxoviruses and reoviruses. Differences in virus size and fine structure (symmetry, presence and/or absence of the envelope and projection size) were used as the criteria for the classification of viruses and thus also for morpho-

logical diagnosis (Tyrrell and Almeida 1967). In some diseases, such as hepatitis or gastroenteritis, none infectious agent was detected by EM; it was not due either to the failure or inefficiency of the method but to the fact that the respective causative viruses are impossible or very difficult to grow in cell cultures. This pitfall was overcome by direct examination of clinical specimens (Almeida 1983; Madeley 1979, 1995). EM is a rapid diagnostic method capable of detecting viruses in a wide range of clinical specimens such as crust, vesicular fluid, lavage fluid, biopsy specimens, feces, urine samples, cerebrospinal fluid, plasma and others (Biel and Gelderblom 1999). The preparation of specimens for EM does not require any special or expensive reagents. EM visualizes all infectious agents that are contained in the analysed specimen regardless of whether or not they are suspected by the clinician (Curry 2003). This method can rapidly detect even very small viruses (Hazelton and Gelderblom 2003).

In plant and animal virology more than 30 000 different viruses have been described, and based on genetic properties, classified into 56 different virus families. Humans have been found to host 21 of the 26 families specific for vertebrates (Van Regenmortel *et al.* 2000). Since the virus families differ from each other in fine structure, an agent visualized by EM can be classified into the relevant family based on morphology. Nevertheless, the development of other techniques, *e.g.*, immunofluorescence, enzyme-linked immunosorbent assay (ELISA), and above all molecular biological methods such as polymerase chain reaction (PCR), progressively reduced the importance of EM in virus diagnosis and microbiology. However, in comparison with other diagnostic methods, EM still benefits from its rapidity and "open view", *i.e.* the capability of detecting all the pathogens present in a clinical specimen (Gentile and Gelderblom 2005). Modern techniques also have limitations: antigenic analysis may not detect antigens that differ in the antigenic make-up while nucleic acid amplification techniques (NAT, PCR) are only capable of detecting the genomic sequences that are already known and PCR can be inhibited by contaminants in clinical specimens. Moreover, commercially available reagents are still lacking. Test kits for many agents have not been marketed yet (Curry *et al.* 2006).

Therefore EM should be utilized as a front line method in infectious diseases emergencies and/or in suspect cases of bioterrorism (Hazelton and Gelderblom 2003; Madeley 2003). If a number of different agents can be etiologically involved, as in gastroenteritis outbreaks, the use of EM will be a prudent decision.

In this review we would like to emphasize the benefit of EM for viral diagnosis and analysis of a wide range of clinical specimens.

2 METHODS

2.1 Specimen collection and pretreatment

Diagnostic EM can be performed when the agent is present in concentrations of a least 10^5 particles per mL (Almeida 1983; Gelderblom *et al.* 1991; Biel *et al.* 2004). In clinical samples, such as vesicles, brain and wart tissues, feces, urine and serum, often much higher particle concern EM was used to analyze various clinical specimens sent to the *National Reference Laboratory for Direct Diagnosis of Viruses, Borreliae and Foreign Cells in Clinical Specimens* (NRL/ELM). Some clinical specimens, such as vesicular fluid, cell culture, urine, serum, plasma, *etc*. were directly analyzed by EM without the need for any previous processing. Sputum specimens or nasopharyngeal secretion should be diluted in PBS and treated with 20 % *N*-acetylcysteine (Gentile and Gelderblom 2005). If suspension turns out negative, the virus may be concentrated by centrifugation, sediment is resuspended in small amount of distilled water. Cerebrospinal fluid usually contains only low concentrations of viruses, so it always needs centrifugation.

To investigate outbreaks of gastroenteritis, feces are prepared as a 10-% suspension in distilled water, followed by low-speed centrifugation to remove cell debris and bacteria (1000 ***g*** for 5 min). After negative staining of supernatants, EM may reveal the presence of one of at least six different virus families involved in diarrhea.

2.2 Requirements for negative staining

Suspensions of viruses must be supported on thin of plastic, carbon, or a combination of the two applied to the surface of an electron microscope grid (Hazelton and Gelderblom 2003; Curry *et al*. 2006); we used 1 % polyvinyl formal (Formvar) with carbon coating film, to enhance its stability. Stability and adhesiveness are best guaranteed using 400 mesh copper grids. To improve the adherence of diagnostic material poly-L-lysine, alcian blue, or UV light may also be used (Gentile and Gelderblom 2005). We preferred coated grids with 1 % poly-L-lysine.

2.3 Negative staining

The negative staining technique developed by Horne and colleagues was a major advance in diagnostic virology (Brenner and Horne 1959; Horne and Wildy 1963, 1979). This technique uses of heavy metals salts to provide contrast to viruses. In EM viruses will appear translucent, while the electrondense stain forms a dense, highly detailed halo. Phosphotungstic acid (PTA) is probably the most commonly used negative stain within diagnostic microbiology, but there are others that are used also. We used routinely 2 % ammonium molybdate and 2 % aqueous uranyl acetate. Two grids were used for each virus suspension, negatively stained one with 2 % ammonium molybdate and with 2 % uranyl acetate. Because uranyl acetate and ammonium molybdate differ in staining properties both stains were applied in parallel of every sample. We have found that PTA destroyed envelope of viruses. The specimens were processed in a biohazard hood in compliance with the biological safety level regulations. The grids were examined under an electron microscope *Joel* 100CX at a magnification of 100000×.

2.4 Immune electron microscopy

Detection sensitivity may be increased and virus identification achieved using the technique of immune electron microscopy, which employs specific antiserum to bind to a viral antigen of interest. There are several immune electron methods (IEM): immune clumping, solid phase immune electron microscopy (SPIEM) and immune-gold labeling. Both immune clumping and SPIEM have routine uses in virology, but immune-gold labeling is mainly used in research investigation.

2.4.1 Immune clumping

Some of the smaller viruses lack distinctive morphological features and are thus difficult to discern from the background debris present in clinical specimens. Target virus, if present, will be aggregated by the antibody into clumps, which are much easier to detect than individual virus particles (Lee *et al*. 1996).

2.4.2 Solid phase immune electron microscopy

The SPIEM methods provides increased sensitivity over immune clumping, allowing detection of virus when only small numbers of particles are present in clinical sample. In SPIEM, antibody-coated grid is floated on drop of the clinical sample under investigation. Virus particles are attracted onto the grid surface and these are spread individually over the grid rather than being aggregated into clumps (Lewis *et al.* 1988).

2.4.3 Immune-gold labeling

Immune-gold labeling utilizes specific antibodies tagged with small particles of colloidal gold as electron dense marker. The technique may be used to localize antigens on whole virus or for example, antigens in thin sections of infected cells or tissues (Rýc *et al.* 1989).

3 DETECTION OF VIRUSES IN VARIOUS CLINICAL SAMPLES

Direct EM detected viruses in a wide range of clinical specimens. It was made possible by the non-selective nature of the method and morphological differences between virus families. Viruses come in whole range of size and shapes, but into three morphological group characterized by (*1*) helical symmetry, (*2*) cubic or icosahedral symmetry, and (*3*) other or complex symmetry (Horne and Wildy 1961; Horne 1974; Nermut 1987; Madeley and Field 1988). Viruses are taxonomically grouped in families and genera by morphological criteria, *i.e.* by size, shape, fine structure of viral capsid and the presence or absence of an envelope and surface projections. Members of the same family or genus have a similar morphological appearance and can originating from various clinical specimens, but they are antigenic distinct. EM detects not only complete but also incomplete viruses and their morphological variants, *e.g*., empty capsids that are found in 30–40 % cells after virus replication (Fig. 1). Antibody-coated viruses or viruses still enclosed in cells are shown in Fig. 2.

3.1 Detection in stool extracts

EM still has an important role in the investigation of viral gastroenteritis, particularly that associated with infants and community outbreaks. The presence of one or more viral species of six families responsible for diarrhea was detected in stool extracts after negative staining (Fig. 3, Table I).

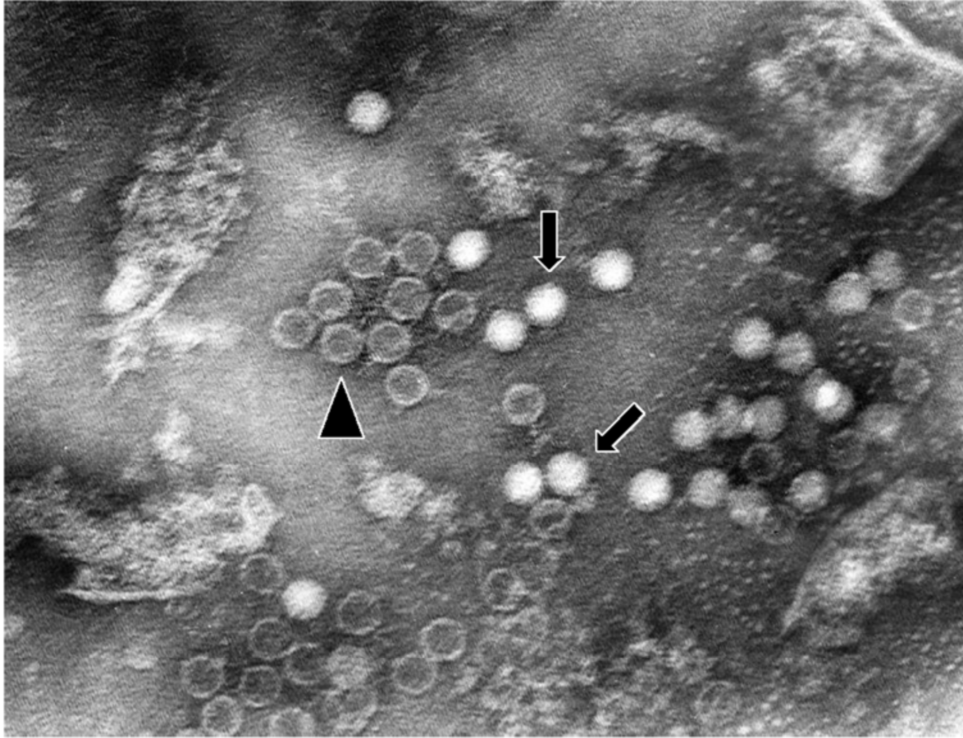

Fig. 1. Complete viruses (*arrows*) and imcomplete viruses (*arrowhead*) of family *Picornaviridae* (100 nm ~ 7 mm).

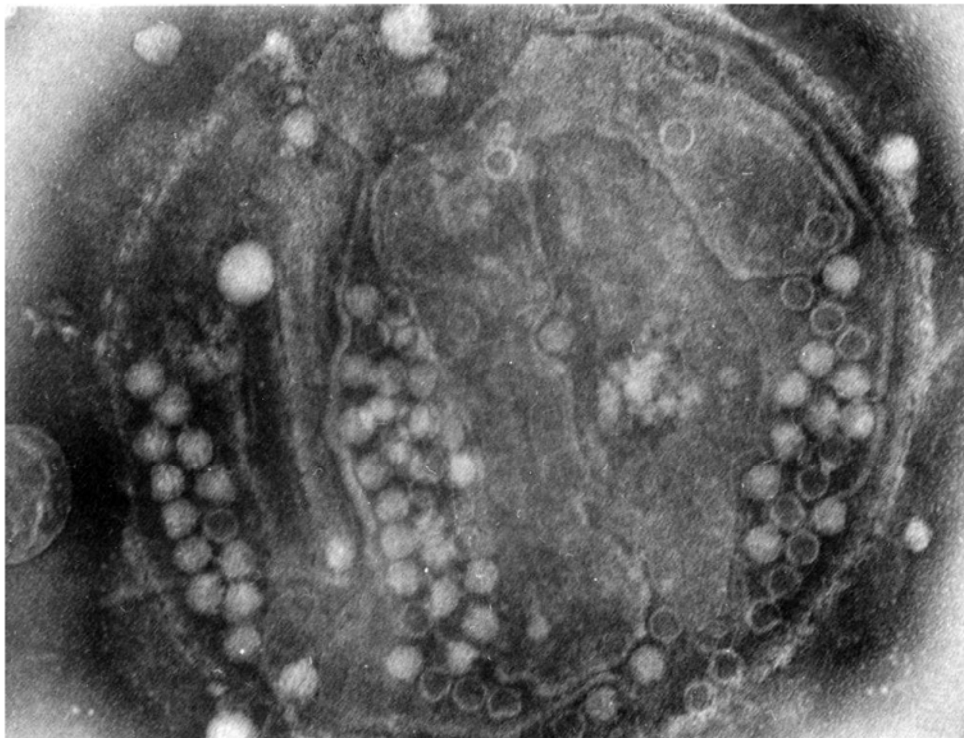

Fig. 2. Picornaviruses still enclosed in a cell (100 nm ~ 13 mm).

Adenoviruses (family *Adenoviridae*) already comprised a large group of antigenically distinct viruses causing wide range symptoms from mild respiratory illness and sore throats to tumors. They are non-enveloped icosahedral viruses with a diameter of ≈80 nm (Fig. 3a). Adenovirus types 40 and 41 are specifically

associated with gastroenteritis (Gary *et al.* 1979). Gastroenteritis originate in adenoviruses are found sporadically throughout the year, being slightly prevalent in winter and spring.

Rotaviruses (family *Reoviridae*) are now being considered to be the most important enteritic viral infection in infancy and are associated with an estimated 600 000 deaths per year, particularly in the developing world (Parashar *et al*. 2003). They are generally spherical, have two capsid layers and are 65–75 nm in diameter which, because of its wheel-like ultrastructure (Fig. 3b), was named 'Rotavirus' (Flewett *et al.* 1973). They were found primarily in stool extracts from young children (less than 5 years of age) and in the elderly in old people's homes and nursing homes. They are isolated more often in winter.

Noroviruses (family *Caliciviridae*) are regarded as the commonest cause of non-bacterial gastroenteritis in developed countries (Kapikian 2000). They are small (≈35 nm) and have icosahedral symmetry with "fuzzy" or ragged edge. They are also called Norwalk-like viruses or small-round-structured viruses (SRSVs) (Mayo 2002), and may account for 68–80 % of acute gastroenteritis outbreaks in all age groups. They are highly infectious, readily transmissible from person to person (Fig. 3c).

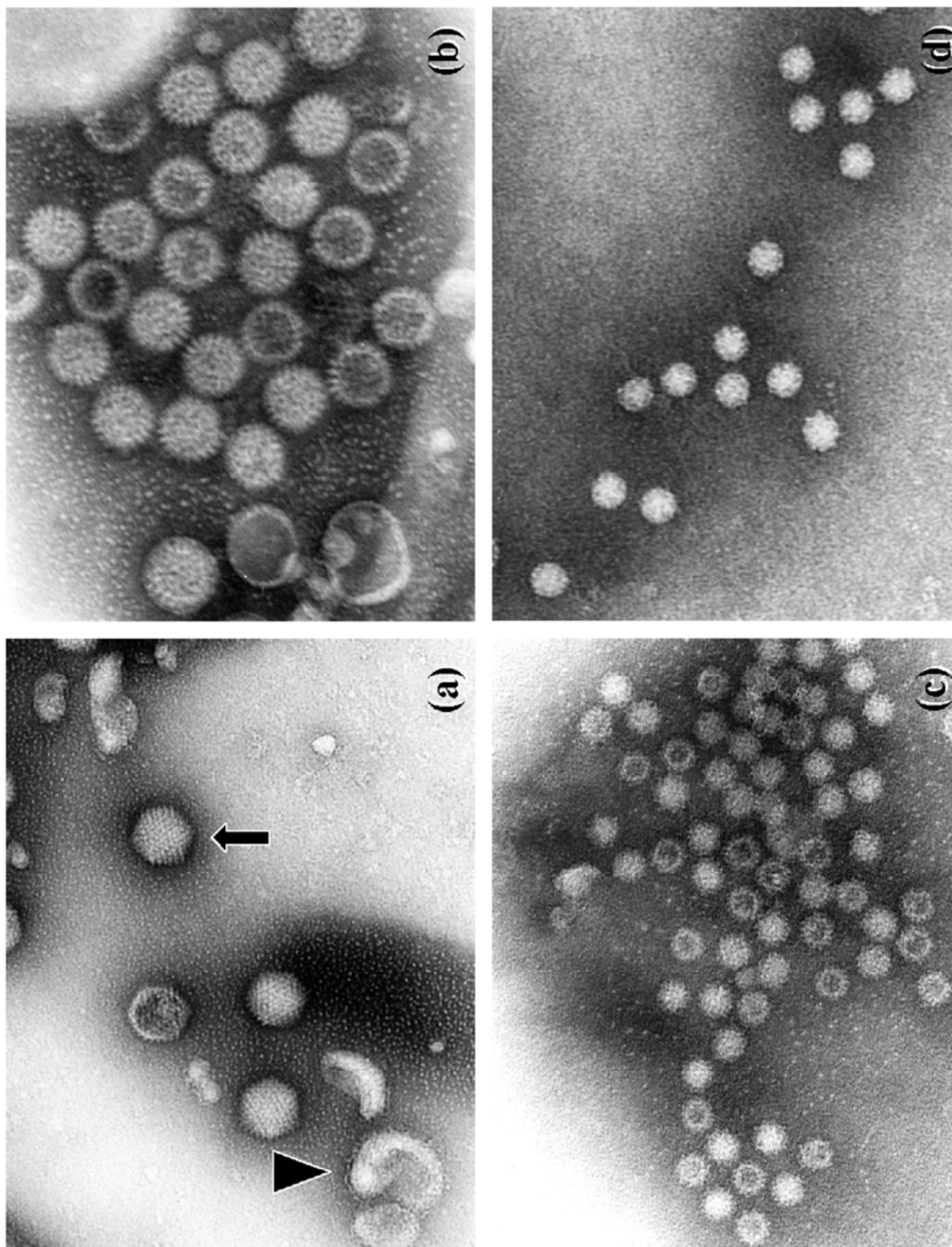

Table I. Viruses detected in 526 positive cases of 700 stool extracts examined in 2004–2008

Virus	Number of cases[a]
Rotaviruses	232 (33)
Adenoviruses	28 (4)
Caliciviruses	246 (35)
Coronaviruses	12 (2)
Astroviruses	8 (1)

[a]Average per year; percentage in *parentheses*.

Sapoviruses (family *Caliciviridae*) are small viruses being ≈32–35 nm in diameter with surface hollows (Madeley and Cosgrove 1976; Kogasaka *et al.* 1981). The viruses were detected mainly in young children, whereas noroviruses infect all age groups (Fig. 3d).

Coronaviruses (family *Coronaviridae*) are spherical, lipid-containing, enveloped particles with tear-drop-shaped surface projections or peplomers. They usually have a diameter excluding projections of between 80 and 120 nm. Coronaviruses cause a wide range of diseases, they are pathogens of the respiratory tract and also of the gastrointestinal tract (Fig. 3a,e).

Astroviruses (family *Astroviridae*) (Appleton and Higgins 1975; Madeley and Cosgrove 1975) are small, morphologically

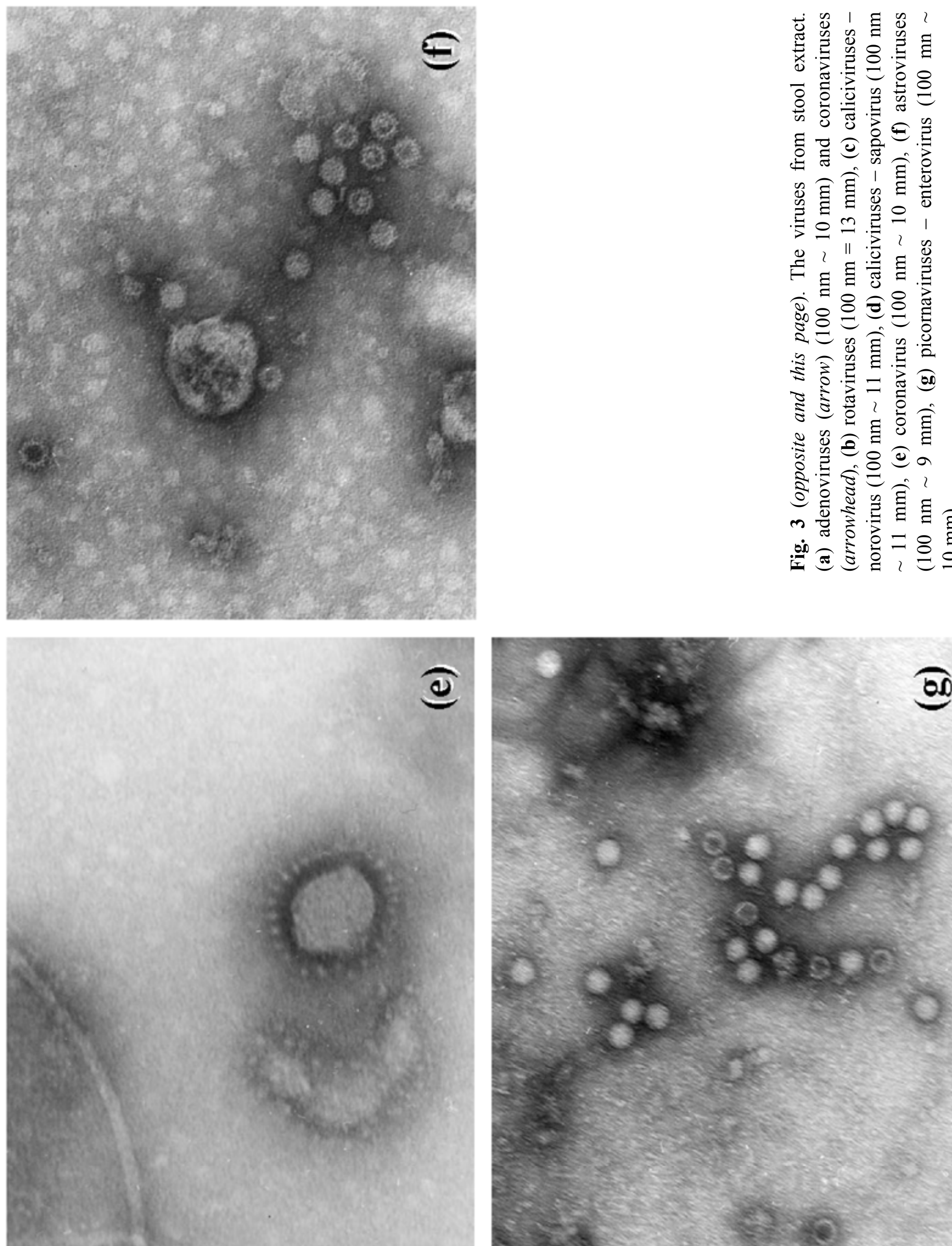

Fig. 3 (*opposite and this page*). The viruses from stool extract. (**a**) adenoviruses (*arrow*) (100 nm ~ 10 mm) and coronaviruses (*arrowhead*), (**b**) rotaviruses (100 nm = 13 mm), (**c**) caliciviruses – norovirus (100 nm ~ 11 mm), (**d**) caliciviruses – sapovirus (100 nm ~ 11 mm), (**e**) coronavirus (100 nm ~ 10 mm), (**f**) astroviruses (100 nm ~ 9 mm), (**g**) picornaviruses – enterovirus (100 mn ~ 10 mm).

distinct from other enteric viruses, being ≈28 nm in diameter with a five or six-pointed surface star and have icosahedral symmetry (Fig. 3f). Human astroviruses cause a less severe diarrhea in infants than rotavirus infection. They are found in children particularly in summer.

Enteroviruses (family *Picornaviridae*) are small non-enveloped viruses with an icosahedral symmetry in general are 25–30 nm in diameter. They rarely cause gastroenteritis, observed predominantly in young children (Fig. 3g).

3.2 Detection in vesicular fluid of skin lesions

Viruses of the family *Herpesviridae* were detected in vesicular fluid of skin lesion. EM still has an important role in the investigation of skin lesions because within 30 min can distinguish herpesviruses from the various poxviruses and papilloma viruses in skin scrapings.

3.3 Detection in cerebrospinal fluid

The non-selective nature of EM was helpful mainly in the analysis of cerebrospinal fluid (CSF). Viruses of the families *Herpesviridae*, *Paramyxoviridae* (Fig. 4a), *Picornaviridae* (Fig. 4b), and *Reoviridae* (orbiviruses) (Fig. 4c), were detected in CSF specimens, varying in quantity, with the last ones being sporadic (Table II).

Table II. Virus abundances in 221 positive cases of 480 cerebrospinal fluids examined in 2004–2008

Virus	Number of cases[a]
Herpesviridae	120 (25)
Picornaviridae	72 (15)
Paramyxoviridae	10 (2)
Adenoviridae	5 (1)
Reoviridae	14 (3)

[a]Average per year; percentage in *parentheses*.

Table III. Virus abundances in 20 positive cases of 60 urine sample examined in 2004–2008

Virus	Number of cases[a]
Polyomaviruses BK	9 (15)
Herpesviridae	6 (10)
Adenoviridae	5 (8)

[a]Average per year; percentage in *parentheses*.

Paramyxoviruses (family *Paramyxoviridae*), which cause measles, mumps and other respiratory infections contain a long, thin helical nucleocapsid 18–22 nm in diameter with a herringbone appearance which is diagnostic feature (Madeley 1997).

Orthomyxoviruses – influenza (family *Orthomyxoviridae*) are identified by the presence of surface spikes, which form characteristic fringe 10 nm long. Particles are usually round or oval with an approximate diameter of 80–120 nm (Madeley 1997).

Picornaviruses (family *Picornaviridae*) are small non-enveloped viruses with icosahedral symmetry, in general are 25–30 nm in diameter. Picornaviruses are broadly classified into four categories: the enteroviruses, the aphthoviruses, the rhinoviruses and the cardioviruses.

3.4 Detection in urine

EM investigation of samples from immunocompromised individuals remains an important use of EM. Urine examination by EM can quickly reveal the presence of polyomavirus (BK) or adenovirus (Cotterill *et al.* 1992; Appleton 2005).

Urine specimens from children and transplant recipients were examined. Apart from viruses of family *Herpesviridae*, polyomaviruses BK (family *Papovaviridae*), adenoviruses (Fig. 5a), and mycoplasmas (Fig. 5b) were detected (Table III). Treatment for organ rejection by inducing further immunosuppression would actually prolong virus replication (Curry *et al*. 2006). Therefore, a rapid diagnosis by EM can significantly aid patient management by indicating the need for the administration of antiviral drugs.

3.5 Investigation of respiratory samples from patients with respiratory symptoms

In respiratory samples by EM were detected picornaviruses (Fig. 6a), orthomyxoviruses (Fig. 6b), and paramyxoviruses (Fig. 6c). Adenoviruses, coronaviruses were also detected (Table IV). Examinations of respiratory samples from patients with severe acute respiratory symptoms, resulted in the discovery of the

SARS, the virus had the characteristic ultrastructural features of a coronavirus (Ksiazek *et al*. 2003; Falsey and Walsh 2003).

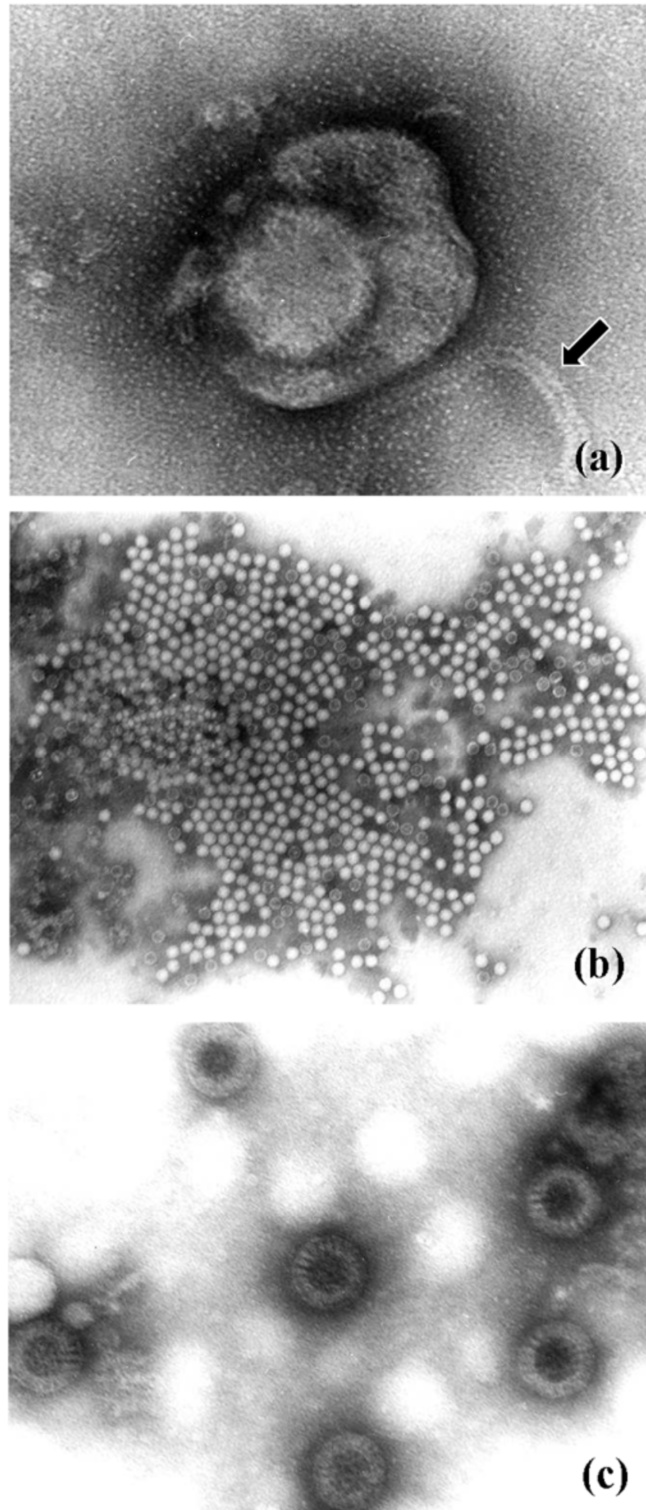

Fig. 4. Viruses from cerebrospinal fluid. (**a**) herpesvirus and fragment of paramyxovirus (*arrow*) (100 nm ~ 36 mm), (**b**) picornaviruses (100 nm ~ 3 mm), (**c**) reoviruses (100 nm ~ 16 mm).

3.6 Detection of viruses in plasma

Plasma examination is more suitable for the detection of viruses from the blood. In the plasma, we detected viruses of the families *Herpesviridae* (Fig. 7), *Picornaviridae*, *Papovaviriadae*, *Paramyxoviridae* and arborviruses. Generally, the detection of these viruses in plasma can be very difficult and requires an experienced specialist.

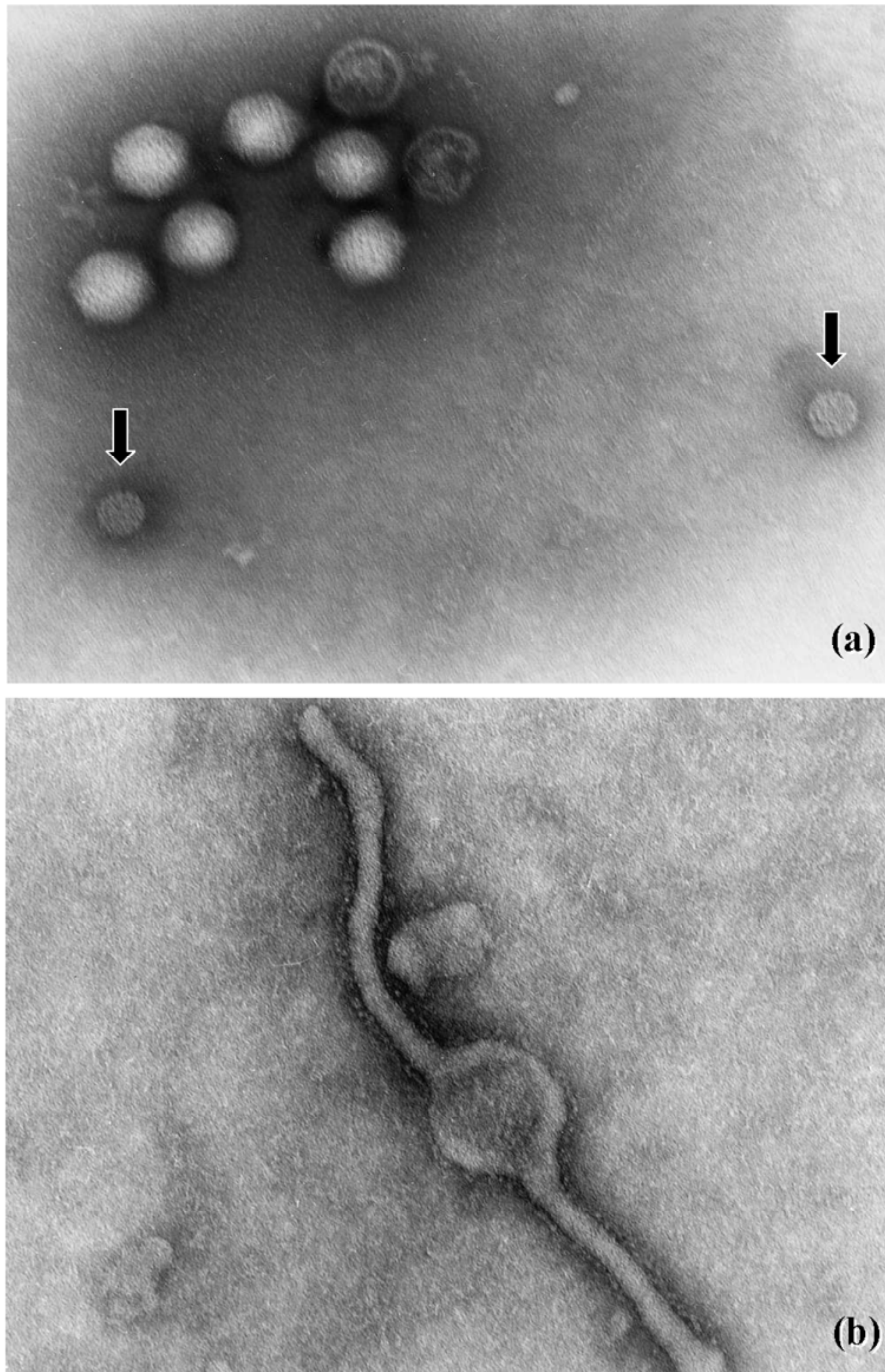

Fig. 5. (**a**) Adenoviruses and polyomaviruses BK (*arrows*) from urine (100 nm ~ 10 mm), (**b**) mycoplasmas from urine (100 nm ~ 5 mm).

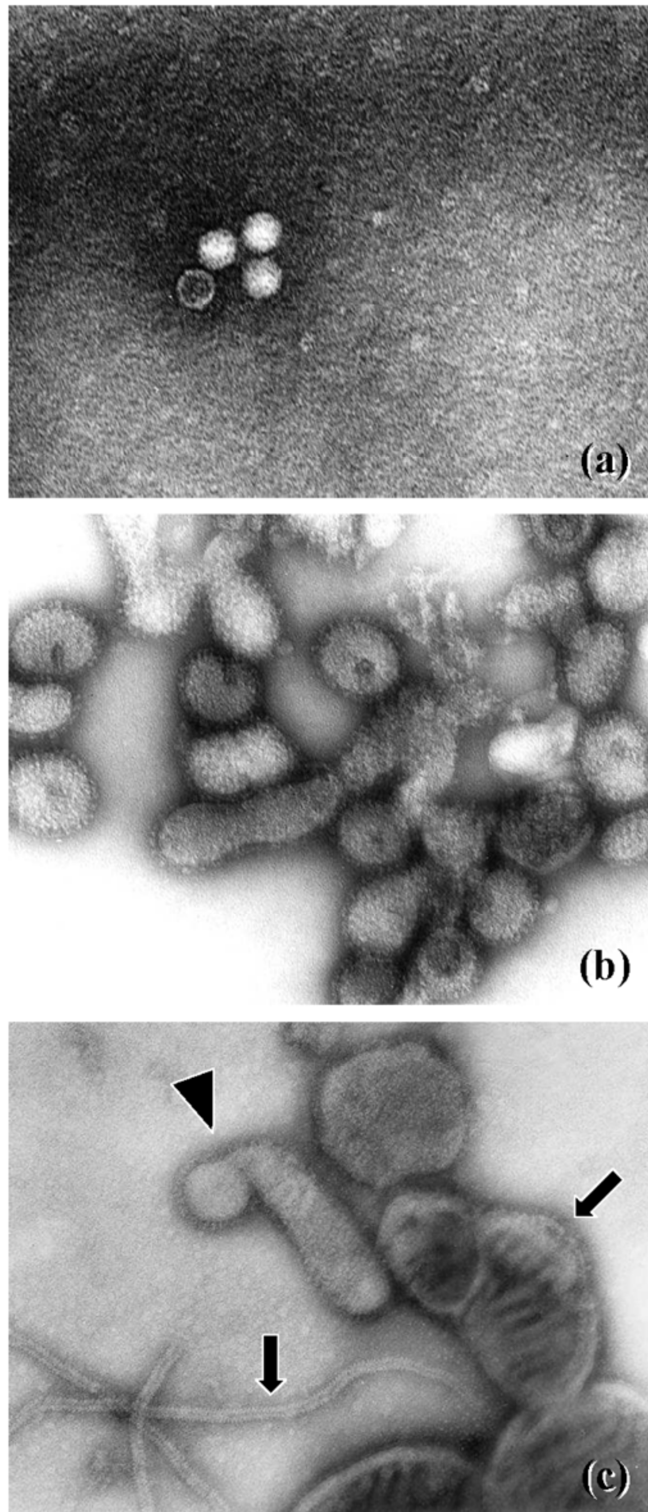

Fig. 6. (**a**) Picornaviruses – rhinovirus (100 nm ~ 13 mm), (**b**) orthomyxovirus – influenza (100 nm ~ 14 mm), (**c**) influenza (*arrows*) and paramyxovirus (*arrowhead*) (100 nm ~ 14 mm).

4 DISADVANTAGES AND ADVANTAGES OF ELECTRON MICROSCOPY

4.1 Disadvantages

Evaluation in EM is time-consuming. Each grid should be screened for 20 min (or 10 fields on the 400 mesh grid) to detect also multiple infections. EM preparation and evaluation cannot be performed in an

automated way, they both are dependent on experienced and dedicated staff. Another limitation is the relatively low sensitivity (10^5–10^6 particles per mL) of diagnostic EM compared to other diagnostic methods. However, as stated before, clinical samples often contain virus concentrations that exceed this limit by several orders. Therefore, the negative evidence is not an absolute diagnosis. A number of effective concentration or immunologic procedures exist that markedly increase sensitivity of EM. Non-immunological procedures include: (*a*) ultracentrifuge concentration, (*b*) agar diffusion, (*c*) direct centrifugation to the electron microscopic grid with the *Beckman* Airfuge (for immunologic procedures *see Chapter 2.4*).

Table IV. Virus abundances in 39 positive cases of 80 nasopharyngeal lavages examined in 2004–2008

Virus	Number of cases[a]
Picornaviridae	16 (20)
Paramyxoviridae	5 (6)
Orthomyxoviridae	6 (8)
Coronaviridae	2 (2)
Adenoviridae	10 (13)

[a]Average per year; percentage in *parentheses*.

4.2 Advantages

As it has been already said, the assets of direct EM are the rapidity and non-selective nature. EM is able to visualize even the smallest viruses. Therefore, the unbiased, "open view" EM is able to detect any virus in a diagnostic sample, *i.e.* also those that were not considered before by the clinician (Biel and Gelderbolm 1999; Gelderblom *et al.* 1991; Hazelton and Gelderblom 2003; Madeley 1995, 2003). In the diagnosis, EM has two advantages over ELISA, PCR and NAT – after a simple and rapid negative staining the EM "open view" makes possible the rapid morphological identification and differential diagnosis of various infectious

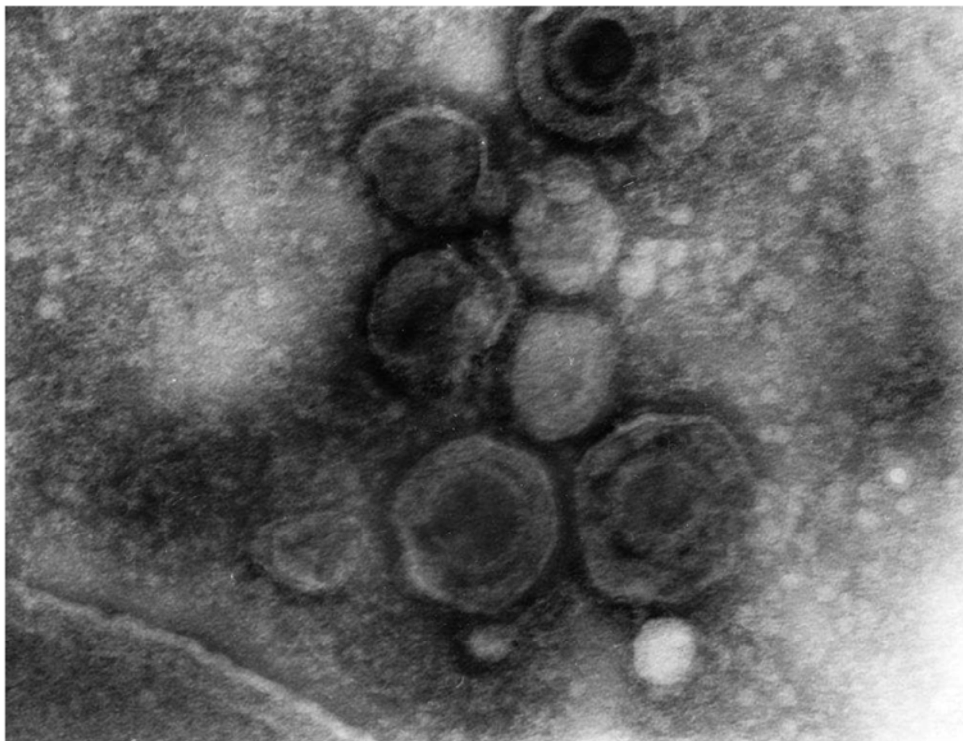

Fig. 7. Viruses of family *Herpesviridae* from plasma (100 nm ~ 29 mm).

agents present in the examined specimen (Table V). The main advantage of the electron microscopy results is that it is based on the morphology and not on the presence of a particular antigen or nucleic acid type. Probably, the intact virions seen with EM may be indicative of infectious virus, whereas detection of antigen or nucleic acid may not always indicate the presence of viable infectious virus particles (Curry 2003).

EM has also been used in many other laboratories for routine diagnosis of human viruses causing gastroenteritis, *i.e.* non-cultivable noroviruses, astroviruses and rotaviruses (Beards 1988; Clark and McKendrick 2004; Frankenhauser *et al.* 2002; Suzuki *et al.* 2005). EM is valuable for differential diagnosis in patients with vesicular dermatitis to exclude smallpox (Hawranek *et al.* 2003; Schimmer *et al.* 2004). Furthermore, it is essential for the diagnosis in outbreaks, epidemics or pandemics caused by an agent, which is

difficult to define (Hazelton and Gelderblom 2003). The causative agent of the recent SARS pandemic was also first identified by EM (Drosten *et al*. 2003; Goldsmith *et al.* 2004; Ksiarek *et al*. 2003). EM was the only method able to identify the causative agent of an outbreak of human monkey pox in the USA 2003 (Reed *et al*. 2004).

Table V. Comparison of the properties and efficiency of different diagnostic tests

Diagnostic test	Specificity	Throughput	Rapidity	Cost/test	Sensitivity
Electron microscopy	morphology only	low	<1 h	inexpensive	medium
Cell culture	any cultivable virus	low	2 d–1 month	expensive	high if cells susceptible
EIA/IF	type/group	high	2 h (IF), 3 h–overnight (EIA)	expensive (IF), inexpensive (EIA)	medium
NAT	high	medium	overnight	expensive	very high
Enzyme assays	specific	low	overnight	medium	low
Serology	type/group	high	have to wait for antibody to be formed	inexpensive	high, if correct antibody is made by patient

EIA – enzyme immunoassay, IF – immunofluorescent antibody, NAT – nucleic acid amplification.

Modern immunological and molecular genetic methods facilitate the routine diagnosis of viruses and thus make it possible for EM to focus on urgent and serious cases of virus infections, *e.g.*, in immunocompromised patients (Biel *et al*. 2004).

Comparison of results obtained by EM and real-time PCR in the detection of viruses from untreated tissue culture showed the superiority of EM that found 38-times higher number of viral copies than PCR (Johnsen *et al.* 2006). EM is occasionally used in combination with other viral methods to identify the causative viruses of zoonotic outbreaks and both seasonal and non-seasonal epidemics or pandemics. For instance, EM visualized reovirus-like particles in the rhesus monkey kidney after inoculation of cerebrospinal fluid and further examination confirmed the diagnosis of reovirus serotype 2 (Hermann *et al.* 2004). The same approach was used to diagnose Chandipura virus implicated in an encephalitis outbreak (Rao *et al.* 2003). EM was helpful in diagnosing a zoonotic disease in a rural population with cowpox-like symptoms in Brazil, caused by vaccinia-like virus called Aracatuba virus (Trinidate *et al*. 2003). In Japan, EM confirmed that the rotavirus infection peak shifted from winter months to early spring months (Suzuki *et al.* 2005).

Recently, noroviruses have become a major problem in hospitals as the cause of gastroenteritis outbreaks (Clark and McKendrick 2004). The great diversity of this group of viruses often leads to misidentification of the causative agent using conventional routine diagnostic RT-PCR kits. In such instances, EM as a complementary method can be of relevance (Galliomore *et al*. 2004; Vipond *et al*. 2004).

EM morphological diagnosis can provide results within 30 min after the specimens is obtained. Viruses present in low amounts can be concentrated by centrifugation. The "open view" makes it possible for EM to detect, in a single specimen, multiple causative agents possibly implicated in acute gastroenteritis (Hazelton and Gelderblom 2003) or neuroinfection. EM is a polyvalent method that can be used for analysis of a wide range of specimens and detection of all types of agents, *e.g.*, *Legionella* in respiratory tract lavage fluid, and can make the routine tissue diagnosis more rapid. The potential of EM can be fully exploited in the quality control, with EM used as a front-line method in parallel with other diagnostic methods (Gentle and Gelderblom 2005). The morphology-based identification coupled with case history often provide sufficient information to the clinician to make the diagnosis, institute therapy and quarantine the patient, if necessary. If further typing of the virus is needed, EM narrows the range of typing tests and thus reduces the costs.

5 CONCLUSIONS

No simple method for an unequivocal and rapid diagnosis of infectious diseases is available (Hazelton and Gelderblom 2003; Madeley 2003). Because the unusual and unexpected can be rapidly identified, EM must remain a front-line method for rapid diagnostic virology, investigation of potential bioterrorist events, and investigation of new and unusual cases of suspected infectious origin. EM has a crucial role to

play in times of the emerging threat of bioterrorism, as it was proved in the case of anthrax letters in the USA (Gentile and Gelderblom 2005). The method is versatile and can be used for different types of samples material and all kinds of agents.

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