O r i g i n a l a r t i c l e

Electron microscopy as a reliable tool for rapid and conventional detection of enteric viral agents: a five-year experience report

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Abstract. *Background and aim of the work:* Since the introduction of the electron microscope and its subsequent development, virology has made a great step forward by the improvement of the basic knowledge on viral structure, as well as by broad application of electron microscopy (EM) to viral diagnosis. In this report, we describe a five-year experience in the use of EM for the diagnosis of enteric viral infections. *Methods:* Three thousand four hundred and ninety stool specimens were analyzed at the Virology Unit (Section of Microbiology, Department of Pathology and Laboratory Medicine, University of Parma, Italy) during a five-year period, from January 1999 to January 2004. The faecal extracts were subjected to EM after negative staining and were simultaneously cultured to evidence the presence of cytopathogenic agents. *Results:* EM directly applied to the above specimens allowed the detection of several enteric viral agents, particularly evidencing those normally hard to cultivate (thus easily lost with culture methods). It also enabled diagnosis of dual gut infections, such as those from rotavirus and calicivirus. On the other hand, EM-based identification of viral agents after cell culture and ultracentrifugation of cytopathogenic agent-containing cellular extracts, allowed the identification of cultivable agents, such as picornaviruses, which can escape the direct EM detection if low concentrated. *Conclusions:* A rationalized use of EM on selected samples, such as stool, appears suitable in epidemiological or clinical conditions when a very rapid diagnosis is required to save time, including cases of suspected emerging viral infections. (www.actabiomedica.it)

Key words: Electron microscopy, rapid diagnosis, enteric viruses

Introduction

The introduction of the first functioning prototype of an electron microscope in the 1930s, and its subsequent development, was a milestone for basic and clinical virology. Particularly with regard to diagnostic virology, the use of electron microscopy (EM), initially limited to rapid diagnosis of the smallpox virus, was extended to «routine» diagnostics in the 1960s, thanks to the introduction of techniques such as negative staining (1-3). Initially used to identify cytopathogenic agents from cell cultures, EM has become even more widely used since its direct application to clinical samples (1, 4). With the implementation of automatic immunoenzymatic methods and, above all, the development of advanced molecular techniques, particularly those based on nucleic acid amplification, the use of EM has significantly decreased (5-7). Many factors have contributed to the marked reduction in EM application to viral diagnosis: its cost and, moreover, its relatively low sensitivity render it not suitable for the screening of a large number of clinical samples.

The present study was carried out on stool specimens at the Virology Unit of the Section of Microbiology (Department of Pathology and Laboratory Medicine, University of Parma, Italy), a comprehensi-

ve centre, offering a combination of different diagnostic tools (EM, cell culture, fluorescence microscopy, nucleic acid amplification methods, antigen and antiviral antibody detection in serum). Its aim was chiefly to underscore the advantages of EM application to the diagnosis of enteric viral infections.

Methods

Virological investigations carried out at the Virology Unit (Section of Microbiology Department of Pathology and Laboratory Medicine, University of Parma, Italy) involved 3490 stool samples, analyzed over a 5-year period, from January 1999 to January 2004.

After the stool sample arrived at the Virology laboratory, a faecal suspension was obtained by diluting specimens to 10% weight/volume in PBS (phosphate buffered saline); the faecal extract was thoroughly emulsified, then centrifuged at low speed (2500 g up to 20 minutes), in order to remove large debris and bacteria. Then the clarified faecal suspension was concentrated by adding polyacrylamide absorbent gel (0.02 gr/550 ml) for about 20 minutes, and subsequently used to prepare a standard drop to be put in contact with a 400-mesh, carbon-reiforced, plastic (formvar)-coated grid. After negative staining using an aqueous solution of phosphotungstic acid (2%, pH 6.4), sample examination was performed using a transmission electron microscope (EM 208S Philips) with a 44,000x magnification. Faecal extracts were simultaneously inoculated into a wide range of cell lines and observed daily under an inverted optical microscope, in order to evidence cytopathogenic agents (conventional cell culture, CC). Positive cell extracts (324) were subjected to EM after ultracentrifugation and negative staining, for final identification steps.

Results

EM and conventional cell culture are routinarily employed in our laboratory for the diagnosis of enteric viral infections. Specifically, EM is used as a rapid method directly on stool samples (which are, in parallel, cultured), as well as on ultracentrifuged cell extracts, which evidenced one or more cytopathogenic agents.

In this study, carried out on 3490 stool specimens analyzed over a five-year period (1999-2004), we evaluated the efficacy of EM in revealing the presence of viral particles, also comparing it to conventional cell culture.

Eight hundred ninety-one (25.5%) of the 3490 studied stool samples were positive for enteric viruses. Positivity for one or more viral agents was determined only by EM in 567 samples (16.2%) and only by CC in 230 samples (6.6%). In 94 samples (2.7%), both methods were able to determine positivity (Table 1). These results clearly outline that EM is the most performant method in evidencing the presence of enteric viruses in stool samples, as summarized in figure 1 (63.6% of the total positive samples, compared to

Table 1. Number and annual distribution of total and positive samples, whose positivity was assessed by the application of electron microscopy (EM) and conventional culture (CC) on stool samples analyzed at the Virology Unit (Section of Microbiology, University of Parma), from January 1999 to January 2004

Year	Total samples	Positive samples		Positive EM*		Positive CC**		Positive [EM + CC]***	
		N.	%	N.	%	N.	%	N.	%
1999	582	139	23.9	86	14.8	48	8.2	5	0.9
2000	706	174	24.6	90	12.7	50	7.1	34	4.8
2001	743	222	29.9	142	19.1	59	7.9	21	2.8
2002	687	160	23.3	106	15.4	38	5.5	16	2.3
2003	689	165	23.9	115	16.7	34	4.9	16	2.3
2004§	83	31	37.3	28	33.7	1	1.2	2	2.4
Total	3490	891	25.5	567	16.2	230	6.6	94	2.7

*: Samples whose positivity was assessed only by EM; **: Samples whose positivity was assessed only by CC; ***: Samples whose positivity was assessed by EM and CC; §: Only January 2004 has been included in this study

25.8% by CC). Indeed, EM allowed us to reveal several cultivable and non-cultivable viral agents, such as rotavirus, adenovirus, picornavirus and calicivirus (Fig. 2).

Specifically, direct electron microscope examination of faecal extracts was very advantageous in detecting, within a few hours after sample arrival, the presence of those viral agents particularly difficult to cultivate (e.g. rotavirus), that would escape identification under parallel CC examination (Table 2, EM). As it can be noted from table 2, rotavirus corresponds to the viral genus most frequently detected in stool (95.6%), as it was expected, taken into account that it represents one of the major cause of paediatric enteric viral infections (8-15).

EM was also useful to evidence the simultaneous presence of two viruses from different families/genera, such as rotavirus and calicivirus (Table 2, EM, 2004). Particularly concerning caliciviruses (then identified as belonging to norovirus genus by molecular methods; see table 2, 2001, 2002, 2004), it is noteworthy that they have "attracted attention" progressively during the last years, being now considered as a relevant cause of outbreaks of gastroenteritis in humans (5, 16), and also potentially involved in nosocomial infections (16-21). The presence of identifiable viral particles in sufficient number to be seen under the electron microscope suggests that, most likely, a significant virus replication has occurred, thus signaling, albeit preliminarly, a possible epidemic wave and allows us to finally identify the viral agent by the appropriate techniques.

On the other hand, the use of conventional culturing (and the subsequent identification of the cytopathogenic agent by means of EM), enabled us to reveal the presence of cultivable adenoviruses, often missed under direct electron microscope observation (Table 2: 64.3% adenovirus by CC, vs 2.9% by EM), probably due to a too low concentration in the stool specimen. The parallel use of the conventional culture method also allowed us to confirm the presence of viruses such as picornaviruses, that might have escaped detection from EM observation of faecal extracts, both because of their poor structural definition and/or a too low concentration in the sample (Table 2: 33.5% picornavirus by CC, vs 0.2% by EM).

Finally, the combined use of EM directly on stool samples and parallel cell culturing (Table 2, EM+CC) allowed us to detect *Reoviridae* viral strains, most likely belonging to the cultivable Orthoreovirus (rather than Rotavirus) genus (1.1%); furthermore, it evidenced the presence of dual gut infections, such as those from rotavirus and adenovirus (18.1%), or rotavirus and picornavirus (6.4%).

Discussion and conclusions

Although EM is not always the method of choice to diagnose viral infections, it has proven to be very

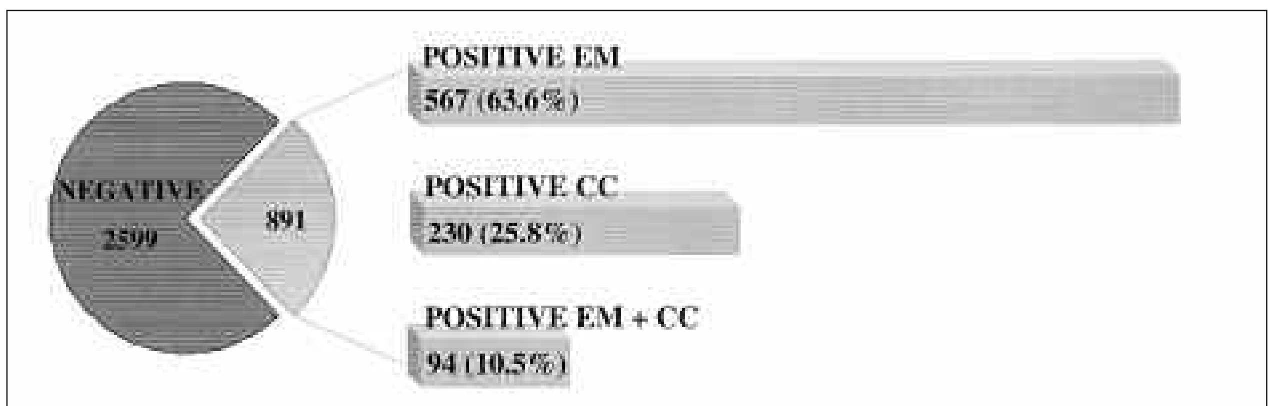

Figure 1. The highest percentage of positive samples obtained by using electron microscopy (EM) directly on faecal extracts ("Positive EM") demonstrates that EM is the most efficient method (vs conventional culture, CC) in revealing viral agents in stool samples analyzed from January 1999 to January 2004

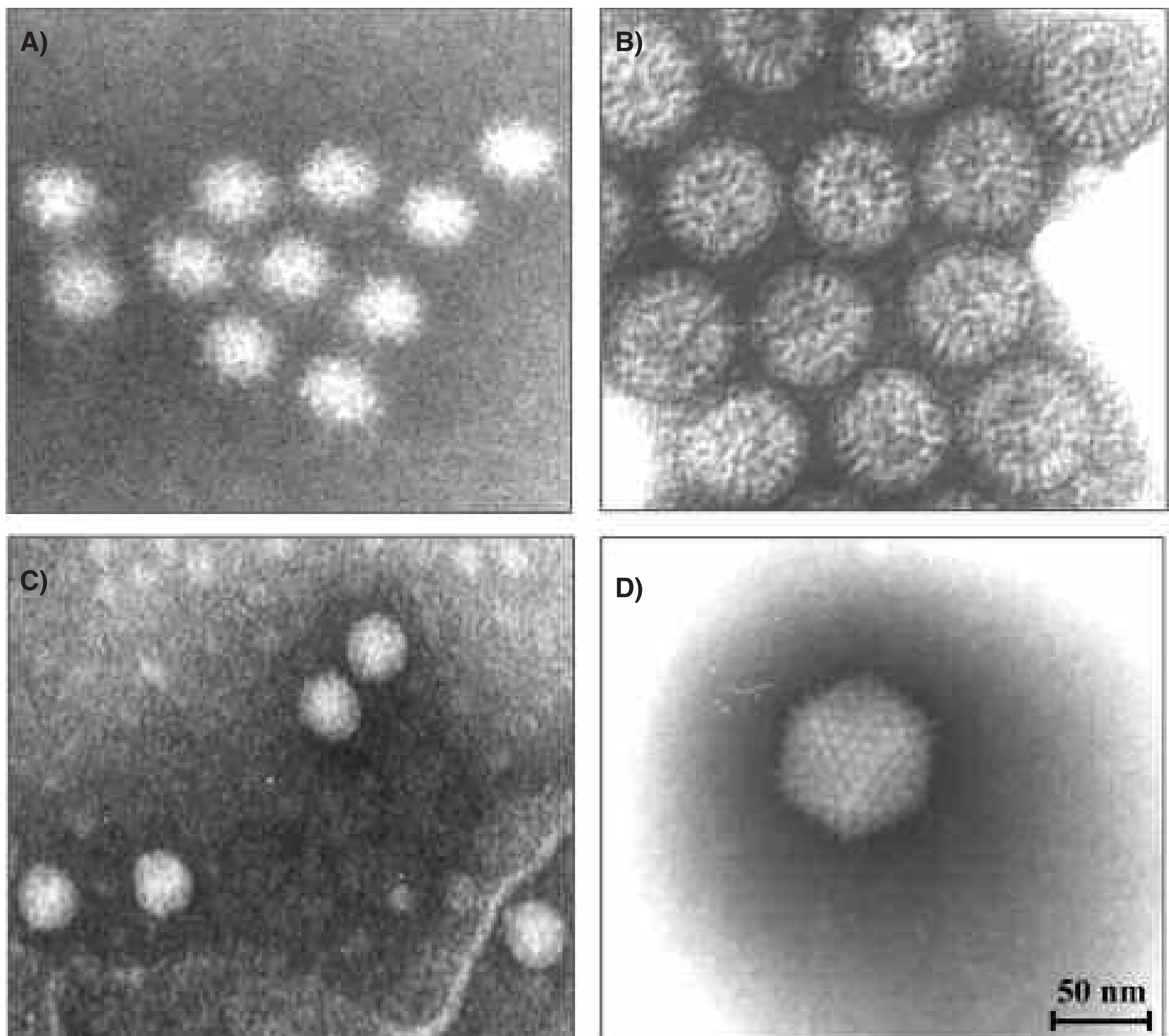

Figure 2. Electron micrographs of enteric viruses after negative staining: A. *Caliciviridae* (Norovirus); B. Rotavirus; C. *Picornaviridae* (Enterovirus); D. Adenovirus. Bar = 50 nm

effective in case of enteric viral infections (22-24). As it clearly appears from this study and is generally agreed, EM offers undoubted advantages linked to the possibility of an easy and very rapid examination of clinical samples and to the direct visualization of viral particles, also allowing us to evidence the concomitant presence of two or more, possibly unexpected, viral agents.

Nevertheless, as already mentioned in the introductive section, the inherent economical limitations and the need for high particle concentration in the sample, as well as the introduction of performant molecular methods, often prevent - or at least strongly reduce - the use of EM as a diagnostic tool.

Indeed, up to date many methodologies are available to detect viruses in clinical specimens, which generally differ in their objectives and principles, indicating the extent to which the choice between one and the other can be at times a matter of opinion and comparison of results and quite hard to make. In fact, very

Table 2. Annual distribution of viral families/genera detected in stool specimens (analyzed from January 1999 to January 2004) by using electron microscopy (EM) and/or conventional culture (CC)

	Year						Total	
	1999	2000	2001	2002	2003	2004§		
EM*	N.	N.	N.	N.	N.	N.	N.	%
Rota	83	86	138	98	112	25	542	95.6
AdV	3	4	2	4	3	1	17	2.9
PrV	0	0	1	0	0	0	1	0.2
CV	0	0	1	4	0	1	6	1.1
Rota/CV	0	0	0	0	0	1	1	0.2
CC**								
Reo	1	2	2	0	0	0	5	2.2
AdV	31	33	38	23	22	1	148	64.3
PrV	16	15	19	15	12	0	77	33.5
[EM+CC]***								
Reo	0	0	0	1	0	0	1	1.1
AdV	2	25	15	11	15	2	70	74.4
Rota/Adv	1	6	6	3	1	0	17	18.1
Rota/PrV	2	3	0	1	0	0	6	6.4

AdV: adenovirus; CV: calicivirus; PrV: picornavirus; Reo: reovirus; Rota: rotavirus.
*: Samples whose positivity was assessed only by EM; **: Samples whose positivity was assessed only by CC; ***: Samples whose positivity was assessed by EM and CC; §: Only January 2004 has been included in this study

often different methods provide complementary rather than "overlapping" information for a comprehensive laboratory diagnosis.

Molecular methods, in particular, are undoubtedly characterized by a greater sensitivity in evidencing the presence of a virus through detection of specific nucleotidic sequences, if compared to EM (1, 5). This latter, however, due to its undirected "open view", is really performant in picking up also new viral particles, as it doesn't need the programmed use of specific probes. Furthermore, a morphological diagnosis, which uncovers the involved viral family (like that performed through EM), often fulfills the physician's immediate needs and can be precious for first emergency measures, until a more precise diagnosis is achieved (25-28).

Thus, the rationalized use of EM on selected samples, i.e. stool specimens, can be advantageous in epidemiological or clinical conditions when very rapid diagnosis is required to save time and avoid complex diagnostic efforts, such as in cases of suspected emerging viral infections and/or when alternative standard diagnostic tools (e.g. cell culture) fail to produce satisfactory results.

References

1. Hazelton PR, Gelderblom HR. Electron microscopy for rapid diagnosis of infectious agents in emergent situations. *Emerg Infect Dis* 2003; 9 (3): 294-303.
2. Biel SS, Madeley D. Diagnostic virology – the need for electron microscopy: a discussion paper. *J Clin Virol* 2000; 22: 1-9.
3. Curry A, Bryden A, Morgan-Capner P, et al. A rationalised virological electron microscope specimen testing policy. PHLS North West Viral Gastroenteritis and Electron Microscopy Subcommittee. *J Clin Pathol* 1999; 52 (6): 471-4.
4. Gelderblom HR, Hazelton PR. Specimen collection for electron microscopy. *Emerg Infect Dis* 2000; 6 (4): 433-4.
5. Rabenau HF, Sturmer M, Buxbaum S, Walczok A, Preiser W, Doerr HW. Laboratory diagnosis of norovirus: which method is the best? *Intervirology* 2003; 46 (4): 232-8.
6. Otsu R, Ishikawa A, Mukae K. Detection of small round structured viruses in stool specimens from outbreaks of gastroenteritis by electron microscopy and reverse transcription-polymerase chain reaction. *Acta Virol* 2000; 44 (1): 53-5.
7. Altindis M, Yavru S, Simsek A, Ozkul A, Ceri A, Koc H. Rotavirus Infection in Children with Acute Diarrhea as Detected by Latex Agglutination, ELISA and Polyacrylamide Gel Electrophoresis. *Indian Pediatr* 2004; 41 (7): 590-4.
8. McIver CJ, Hansman G, White P, Doultree JC, Catton M, Rawlinson WD. Diagnosis of enteric pathogens in children with gastroenteritis. *Pathology* 2001; 33 (3): 353-8.

9. Dennehy PH, Nelson SM, Spangenberger S, Noel JS, Monroe SS, Glass RI. A prospective case-control study of the role of astrovirus in acute diarrhea among hospitalized young children. *J Infect Dis* 2001; 184 (1): 10-5.
10. Kasule M, Sebunya TK, Gashe BA, Armah G, Steele AD. Detection and characterization of human rotavirus among children with diarrhoea in Botswana. *Trop Med Int Health* 2003; 8 (12): 1137-42.
11. Steele D, Reynecke E, de Beer M, Bos P, Smuts I. Characterization of rotavirus infection in a hospital neonatal unit in Pretoria, South Africa. *J Trop Pediatr* 2002; 48 (3): 167-71.
12. Iturriza Gomara M, Kang G, Mammen A, Jana AK, et al. Characterization of G10P[11] rotaviruses causing acute gastroenteritis in neonates and infants in Vellore, India. *J Clin Microbiol* 2004; 42 (6): 2541-7.
13. Piednoir E, Bessaci K, Bureau-Chalot F, et al. Economic impact of healthcare-associated rotavirus infection in a paediatric hospital. *J Hosp Infect* 2003; 55 (3): 190-5.
14. Sharma R, Hudak ML, Premachandra BR, et al. Clinical manifestations of rotavirus infection in the neonatal intensive care unit. *Pediatr Infect Dis J* 2002; 21 (12): 1099-105.
15. Rodriguez-Baez N, O'Brien R, Qiu SQ, Bass DM. Astrovirus, adenovirus, and rotavirus in hospitalized children: prevalence and association with gastroenteritis. *J Pediatr Gastroenterol Nutr* 2002; 35 (1): 64-8.
16. Froggatt PC, Barry Vipond I, Ashley CR, Lambden PR, Clarke IN, Caul EO. Surveillance of norovirus infection in a study of sporadic childhood gastroenteritis in South West England and South Wales, during one winter season (1999-2000). *J Med Virol* 2004; 72 (2): 307-11.
17. Lynn S, Toop J, Hanger C, Millar N. Norovirus outbreaks in a hospital setting: the role of infection control. *N Z Med J* 2004; 117(1189): U771.
18. Meyer E, Ebner W, Scholz R, Dettenkofer M, Daschner FD. Nosocomial outbreak of norovirus gastroenteritis and investigation of ABO histo-blood group type in infected staff and patients. *J Hosp Infect* 2004; 56 (1): 64-6.
19. McCall J, Smithson R. Rapid response and strict control measures can contain a hospital outbreak of Norwalk-like virus. *Commun Dis Public Health* 2002; 5 (3): 243-6.
20. Gonzalez Moran F, Moreno Civantos A, Mateo Ontanon Sd S, et al. Nosocomial epidemic outbreak of acute gastroenteritis by Norwalk-like virus. *Med Clin* (Barc) 2002; 118 (16): 611-5.
21. Billgren M, Christenson B, Hedlund KO, Vinje J. Epidemiology of Norwalk-like human caliciviruses in hospital outbreaks of acute gastroenteritis in the Stockholm area in 1996. *J Infect* 2002; 44 (1): 26-32.
22. Leung WK, To KF, Chan PK, et al. Enteric involvement of severe acute respiratory syndrome-associated coronavirus infection. *Gastroenterology* 2003; 125 (4): 1011-7.
23. Mori I, Matsumoto K, Sugimoto K, et al. Prolonged shedding of rotavirus in a geriatric patient. *J Med Virol* 2002; 67 (4): 613-5.
24. Marshall J, Botes J, Gorrie G, et al. Rotavirus detection and characterisation in outbreaks of gastroenteritis in aged-care facilities. *J Clin Virol* 2003; 28 (3): 331-40.
25. Riordan FA, Quigley T: Estimating hospital admissions due to rotavirus gastroenteritis from hospital episode statistics. *J Infect* 2004; 49 (1): 13-6.
26. Rivest P, Proulx M, Lonergan G, Lebel MH, Bedard L. Hospitalisations for gastroenteritis: the role of rotavirus. *Vaccine* 2004; 22 (15-16): 2013-7.
27. Roman Riechmann E, Wilhelmi de Cal I, Cilleruelo Pascual ML, Calvo Rey C, Garcia Garcia ML, Sanchez-Fauquier A. Nosocomial gastroenteritis and asymptomatic rotavirus and astrovirus infection in hospitalized children. *An Pediatr (Barc)* 2004; 60(4): 337-43.
28. Gelber SE, Ratner AJ. Hospital-acquired viral pathogens in the neonatal intensive care unit. *Semin Perinatol* 2002; 26 (5): 346-56.

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