Comparison of Three Electron Microscopy Techniques for the Detection of Human Rotaviruses

Pierre Trépanier,* Robert Alain, Valeriu Micusan, Bernadette McLaughlin, and Laurent Berthiaume

*Université du Québec, Centre de Recherche en Virologie, and Centre de Recherche en Immunologie, Institut Armand-Frappier, C.P. 100, Laval, Québec, Canada, H7N 4Z3, and Laboratoire de Microbiologie, Hôpital Ste-Justine, Montréal, Canada

Abstract Rotavirus detection by direct electron microscopy was compared with direct and indirect immune electron microscopy techniques. The latter two approaches permitted the enumeration of 25 and 103 times more rotaviruses respectively, than direct electron microscopy. Also, 70% and 90% of the virus particles were aggregated by direct and indirect immune electron microscopy techniques respectively, thus facilitating their detection.

Diagnostic electron microscopy of feces has proved very successful in the last few years in the detection of several non-cultivable viruses among which some represented new viral entities. In that regard, the Norwalk agent (12), rotaviruses (10), coronaviruses (6), astroviruses (14), and caliciviruses (15) were all visualized by electron microscopy from cases of gastroenteritis. However, it has been shown in numerous studies that rotaviruses were the most common viral agents seen in infantile gastroenteritis. Nowadays both direct electron microscopy (DEM) and immune electron microscopy (IEM) are used in the detection of rotaviruses (3, 18). We have previously reported two IEM techniques helpful in detecting human rotaviruses from infantile gastroenteritis feces (4, 5). Here we extend our study by comparing the sensitivity of these two latter IEM techniques with the commonly used DEM.

MATERIALS AND METHODS

Nature and collection of specimens. Infantile gastroenteritis feces were kindly provided by the Laboratory of Microbiology, Ste-Justine Hôpital, Montréal, Canada.

Clarification of specimens. Feces were prepared for electron microscopy as a 20% (wt/vol) suspension made in phosphate-buffered saline (PBS). This suspension was then clarified at $1,000 \times g$ for 5 min and $10,000 \times g$ for 20 min and

supernatants used directly. Ten feces positive for rotaviruses were randomly selected for this study.

Electron microscopy techniques. The following approaches were used with the clarified supernatants:

1) Direct examination or direct electron microscopy (DEM). A drop of sample deposited on the grid was first blotted after 30 sec with the edge of a filter paper. Similarly, a drop of 3% phosphotungstic acid (PTA), pH 6.0, was then deposited on the grid for 30 sec and blotted before examination. This technique was employed as a control standard procedure because it is commonly used.

2) Human gamma-globulins-in-agar method (Agar+IgG). The sample (25 μl) was deposited on the surface of 2% agar containing 1:25 PBS-diluted pools of commercially available human gamma-globulins (4). This dilution was shown to be optimal for rotavirus aggregation, without heavily masking the viral structures. A grid placed, face downward, on top of the drop was picked up after almost complete diffusion of the aqueous phase into the agar and PTA stained before examination.

3) Indirect IEM with ferritin-labeled antibodies (IEM-Ft). Previously described in detail (5), this approach briefly consisted of first mixing the sample (30 μl) for 15 min at room temperature with 10 μl of rabbit anti-bovine rotavirus serum diluted 1:40 in PBS. To this suspension was then added for another 15 min 10 μl of 1:50 PBS-diluted ferritin-labeled goat anti-rabbit IgG. The whole mixture was subsequently deposited in a microtiter well half filled with 1% agar. As in the Agar+IgG technique, a grid placed on top of the drop was then eventually picked-up and PTA stained before examination.

Carbon formvar coated 300 mesh copper grids were used throughout this work. Grids that were hydrophobic were rejected. For each sample, isolated or aggregated virus particles were enumerated on five randomly selected grid squares using a Philips EM 300 electron microscope at 80kV.

RESULTS

After enumerating the rotaviruses on five grid squares per specimen using the three different approaches, the compiled results showed that virus counts were lower with the DEM approach. In order to allow a better comparison between the techniques' sensitivity, the results from the latter technique were transformed into unitary values. Then, results from other techniques (Agar+IgG, IEM-Ft) were compared with DEM and the different ratios obtained were averaged for each technique and are represented in Table 1. The compared relative sensitivity of the different techniques indicated that IEM techniques have permitted the detection of a greater number of rotaviruses per grid square. The Agar+IgG and IEM-Ft techniques revealed nearly 25 and 103 times, respectively, more virus particles per square grid than DEM. Moreover, IEM techniques were very successful in complexing the rotaviruses as also shown in Table 1. In our study, approximately 93% of the particles were seen in isolation with DEM while 71%

Table 1. Comparison of three electron microscopy techniques
in the detection of human rotavirus[a]

Techniques	Relative sensitivity	Standard deviation	% virus ≥ 2[e]	Standard deviation
DEM[b]	1	0	6.6	10.6
Agar + IgG[c]	23.6	23.6	71	21.6
IEM-Ft[d]	103	105	91.5	10.8

[a] Five grid squares were enumerated per sample; data represent average values per grid square of 10 specimens.
[b] Direct electron microscopy.
[c] Agar (2%) containing human gamma-globulins.
[d] Indirect immune electron microscopy using ferritin-labeled antibodies.
[e] Percentage of rotaviruses seen in clumps of 2 or more particles.

Table 2. Sensitivity of electron microscopy techniques
analyzed by the Student's test for paired data[a]

Techniques	Agar + IgG[c]	IEM-Ft[d]
DEM[b]	0.004	0.001
Agar + IgG	—	0.04

[a] Critical region ≤ 0.05.
[b] Direct electron microscopy.
[c] 2% agar containing human gamma-globulins.
[d] Indirect immune electron microscopy using ferritin-labeled antibodies.

and 92% of the rotaviruses were specifically clumped by the Agar+IgG and IEM-Ft techniques, respectively. The results used in the construction of Table 1 were furthermore transformed into logarithmic $(x+1)$ values to facilitate data processing and statistically analyzed by the Student's test for paired data. Table 2 shows that the relative sensitivity of all paired techniques tested statistically rejected the similitude of paired data (critical region ≤ 5%), indicating a significant difference between the techniques' sensitivity.

Isolated rotaviruses seen by DEM are presented in Fig. 1A. However, rotavirus detection and identification may prove more difficult when dealing in some samples with isolated altered particle (Fig. 1B, C). Aggregated rotaviruses are, however, more easily detected on the grid. Figure 1D shows an immune complex of rotaviruses seen after using the Agar+IgG method. Altered particles or fragments of virions were also frequently seen within such aggregates as shown in Fig. 1E. The indirect IEM approach also permitted an easy detection of rotaviruses because of the ferritin molecules surrounding the immune complexes (Fig. 1F) which greatly increased the electron density of the aggregates thus permitting their detection at very low magnification. Single shelled human rotaviruses exposing their group antigens were aggregated, as expected, by the antibovine rotavirus serum but some enveloped particles were also associated with the immune complexes, presumably because of a partial disruption of their outer membrane.
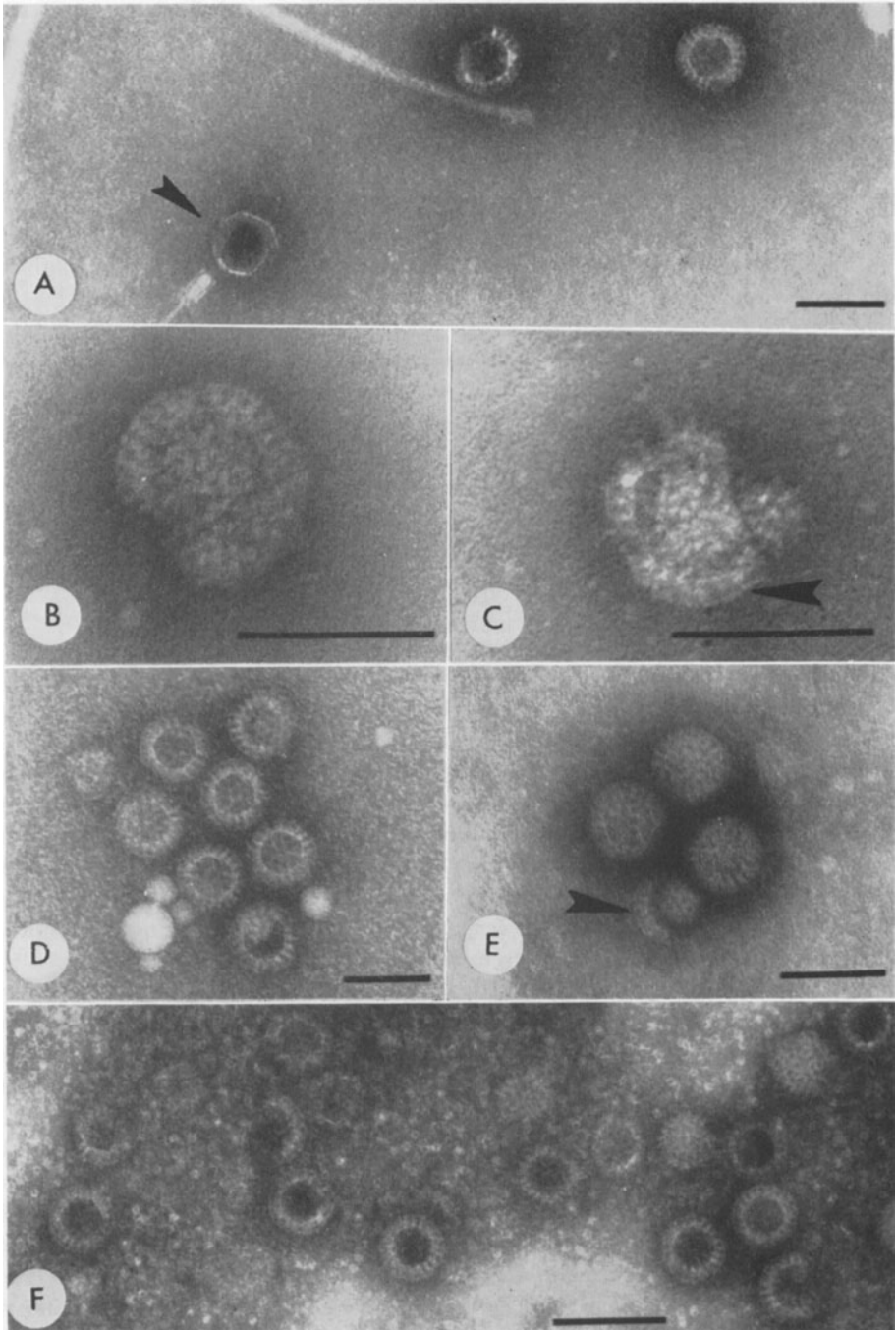
Fig. 1

DISCUSSION

Since human rotaviruses are not yet amenable to tissue culture isolation, DEM has become a widely used approach for diagnostic purposes. Other techniques such as counter-immunoelectro-osmophoresis (16), radioimmunoassay (11) or enzyme-linked immunosorbent assay (21) have also been used in the detection of rotaviruses. However, the possibility of visualizing other viruses by electron microscopy greatly favors this approach in the cases of gastroenteritis (19). Our study indicates that IEM techniques revealed per surface area much more rotaviruses than DEM. Other workers have also previously reported on the greater sensitivity of IEM techniques over DEM (2, 7, 8, 13, 17). With plant viruses, Milne and Luisoni (17) and Derrick (7) reported increases of 10 to 50 times more particles on grid per surface area after using IEM techniques. Similarly, the Agar+IgG method has permitted us to detect 25 times more rotaviruses than DEM. Also, the indirect approach using ferritin-labeled antibodies proved approximately one-hundred times more sensitive than DEM. A few studies have previously dealt with diagnostic indirect IEM techniques (9, 20). In one of the latter studies, Edwards et al (9) have found that indirect IEM after using serial dilutions was more sensitive than direct IEM. Although the evaluations of sensitivity of the techniques were different, our study also agreed with this finding, the IEM-Ft approach being more sensitive than the Agar+IgG method by a factor of about four.

The simplicity, rapidity and sensitivity associated with the Agar+IgG method make it a recommendable technique that can be routinely used for the detection of rotaviruses. Aggregation of rotaviruses in which this technique excelled has facilitated rotavirus detection. Furthermore, other viruses as well can be detected using this approach because the agar contains antibodies against a variety of viruses (4). However, when ambiguous results are expected because of poorly preserved samples and/or very low virus concentration eliminating virus aggregation by the Agar+IgG method, the indirect IEM-Ft technique may prove very useful. The higher sensitivity of the latter approach together with the ferritin molecules serving as a reliable marker are likely to facilitate rotavirus detection and identification. Also, this approach can be adapted to other viruses (5). The latter technique may also represent an alternative procedure to the use of intact viral particles as IEM markers for the identification of structures or components with no recognizable morphology, but antigenically related to known viruses as recently reported by Almeida et al (1).

Fig. 1. A) Isolated rotavirus seen by direct electron microscopy; also shown is a phage (arrow) easily differentiated from rotaviruses. Careful examination may prove essential in the cases of slightly altered (B) or heavily damaged (C) virus particles. In the latter case, a fragment of the double membrane (arrow) represents the only recognizable rotavirus structure. D) Aggregated rotaviruses seen with the Agar+IgG technique; collapsed particles are frequently seen within aggregates. (E, arrow). F) Typical immune complex of rotaviruses heavily tagged with ferritin molecules after using the IEM-Ft technique.

REFERENCES

1) Almeida, J.D., Skelly, J., Howard, C.R., and Zuckerman, A.J. 1981. The use of markers in immune electron microscopy. J. Virol. Methods 2: 169–174.

2) Anderson, N., and Doane, F.W. 1973. Specific identification of enteroviruses by immuno-electron microscopy using a serum-in-agar diffusion method. Can. J. Microbiol. 19: 585–589.

3) Banatvala, J.E., Chrystie, I.L., and Totterdell, B.M. 1978. Rotaviral infections in human neonates. Javma 173: 527–530.

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

5) Berthiaume, L., Micusan, V., Alain, R., and Trépanier, P. 1981. Rapid identification of viruses by a simple indirect immune electron microscopy technique using ferritin-labelled antibodies. J Virol. Methods 2: 367–373.

6) Caul, E.D., Paver, W.K., and Clarke, S.K.R. 1975. Coronavirus particles in faeces from patients with gastroenteritis. Lancet I: 1192.

7) Derrick, K.S. 1973. Quantitative assay for plant viruses using serologically specific electron microscopy. Virology 56: 652–653.

8) Devine, R.D., and Lee, V.P. 1975. The use of polyelectrolytes and immuno-electron microscopy for the detection of enterovirus in stools. Can. J. Public Health 66: 43.

9) Edwards, E.A., Valters, W.A., Boehm, L.G., and Rosenbaum, M.J. 1975. Visualization by immune electron microscopy of viruses associated with acute respiratory disease. J. Immunol. Methods 8: 159–168.

10) Flewett, T.H., Bryden, A.S., and Davies, H. 1973. Virus particles in gastroenteritis. Lancet II: 1497.

11) Kalica, A.R., Purcell, R.H., Sereno, M.M., Wyatt, R.G., Kim, H.W., Chanock, R.M., and Kapikian, A.Z. 1977. A microtiter solid phase radioimmunoassay for detection of the human reovirus-like agent in stools. J. Immunol. 118: 1275–1279.

12) Kapikian, A.Z., Wyatt, R.G., Dolin, R., Thornhill, T.S., Kalica, A.R., and Chanock, R.M. 1972. Visualization by immune electron microscopy of a 27-NM particle associated with acute infectious nonbacterial gastroenteritis. J. Virol. 10: 1075–1081.

13) Kelen, A.E., Hathaway, A.E., and McLeod, D.A. 1971. Rapid detection of Australia/SH antigen and antibody by a simple and sensitive technique of immunoelectron microscopy. Can. J. Microbiol. 17: 993–1000.

14) Madeley, C.R., and Cosgrove, B.P. 1975. Viruses in infantile gastroenteritis. Lancet II: 124.

15) Madeley, C.R., and Cosgrove, P.B. 1976. Caliciviruses in man. Lancet I: 199–200.

16) Middleton, P.J., Petric, M., Hewitt, C.M., Szymanski, M.T., and Tam, J.S. 1976. Counter-immunoelectro-osmophoresis for the detection of infantile gastroenteritis virus (orbi-group) antigen and antibody. J. Clin. Pathol. 29: 191–197.

17) Milne, R.G., and Luisoni, E. 1975. Rapid high-resolution immune electron microscopy of plant viruses. Virology 68: 270–274.

18) Nicolaieff, A., Obert, G., and Regenmortel, N.H.V. van. 1980. Detection of rotaviruses by serological trapping on antibody-coated electron microscope grids. J. Clin. Microbiol. 12: 101–104.

19) Spratt, H.C., Marks, M.I., Gomersall, M., Gill, P., and Pai, C.H. 1978. Nosocomial infantile gastroenteritis associated with minirotavirus and calicivirus. J. Pediatr. 93: 922–926.

20) Valters, W.A., Boehm, L.G., Edwards, E.A., and Rosenbaum, M.J. 1975. Detection of adenovirus in patient specimens by indirect immune electron microscopy. J. Clin. Microbiol I: 472–475.

21) Yolken, R.H., Kim, H.W., Clem, T., Wyatt, R.G., Chanock, R.M., Kalica, A.R., and Kapikian, A.Z. 1977. Enzyme-linked immunosorbent assay (ELISA) for detection of human reovirus-like agent of infantile gastroenteritis. Lancet II: 263–267.