JOURNAL OF CLINICAL MICROBIOLOGY, May 1990, p. 864–868
0095-1137/90/050864-05$02.00/0

Vol. 28, No. 5

Sensitive Solid-Phase Immune Electron Microscopy Double-Antibody Technique with Gold-Immunoglobulin G Complexes for Detecting Rotavirus in Cell Culture and Feces

BINGREN WU,[1] JAMES B. MAHONY,[2,3]* GERARD SIMON,[3] AND MAX A. CHERNESKY[2,3]

Department of Virology, Inner Mongolia Anti-Epidemic Station, Huhehote, People's Republic of China[1]; McMaster University Regional Virology Laboratory, St. Joseph's Hospital, Hamilton, Ontario, Canada L8N 4A6[2]; and Department of Pathology, McMaster University, Hamilton, Ontario, Canada L8N 3Z5[3]*

Received 11 September 1989/Accepted 6 January 1990

A new solid-phase immune electron microscopy double-antibody colloidal-gold technique (SPIEMDAGT) was developed and compared with direct electron microscopy, direct immune electron microscopy, and enzyme immunoassay for detecting rotavirus. Guinea pig and rabbit antirotavirus antisera were used as capture and detector antibodies, respectively, and goat anti-rabbit immunoglobulin G-gold complexes were employed as a label. Animal rotavirus in cell culture media and human virus in stool specimens were detected by this method. On average, SPIEMDAGT detected 800 times more virus particles than direct electron microscopy and 45 times more particles than direct immune electron microscopy and yielded 20% more positives than enzyme immunoassay. SPIEMDAGT could detect not only viral antigen associated with morphologically recognizable particles but also antigen present when whole virus particles were not visible.

Rotavirus is an important cause of infectious gastroenteritis, not only in infants but also in young children. The human rotavirus does not propagate in conventional cell cultures. Therefore, prior to the development of enzyme immunoassay (EIA) techniques, direct electron microscopy (DEM) with negative staining was the only method of providing a definitive diagnosis. The major disadvantage of DEM is that when rotavirus particles are present in low concentrations ($<10^6$ particles per ml) in stool specimens, the virus is difficult to detect. Various methods of immune electron microscopy (IEM) have been developed to overcome this limitation. IEM is based on the formation of aggregates that occurs when viral particles are mixed with an antirotavirus antibody. Although the IEM technique has facilitated the detection of rotavirus and is considerably more sensitive than DEM, IEM is dependent upon optimal concentrations of antibody and antigen and is susceptible to a prozone phenomenon (3). Derrick (2) described an improved IEM method employing a solid phase in which grids were coated with antibody and used for specific trapping of the virus. This method minimized or eliminated the prozone phenomenon and was later modified by precoating grids with staphylococcal protein A to anchor antiviral antibodies for the detection of rotavirus (9, 10). Milne and Luisoni (8) attempted to further improve virus detection by adding a second layer of decorator antibody to captured virus, which produced a halo (decoration) around the particles. More recently, gold-labeled antibody has been introduced to IEM to enhance the visibility of viruses (4) and has been successfully used for the detection of gastroenteric animal viruses by an indirect technique (7). This article reports a solid-phase immune electron microscopy double-antibody colloidal-gold technique (SPIEMDAGT), which is significantly more sensitive than DEM, IEM, and EIA for the detection of human rotavirus.

* Corresponding author.

MATERIALS AND METHODS

Virus. Simian rotavirus SA11 was kindly supplied by Francis Doane and Nan Anderson, University of Toronto, Toronto, Canada, and was prepared as described previously (6). The infectivity titer of simian rotavirus SA11 was 7.1×10^7 50% tissue culture infective doses per ml.

Specimens. Stool specimens collected from patients with gastroenteritis were sent to the McMaster University Regional Virology Laboratory at St. Joseph's Hospital, Hamilton, Ontario, Canada. They were processed to 10% suspensions in phosphate-buffered saline (PBS), pH 7.2, and were centrifuged at $3,000 \times g$ for 30 min. There were 20 specimens positive for rotavirus by EM or by either Pathfinder Rotavirus (Kallestad, Austin, Tex.) or Testpack Rotavirus (Abbott Laboratories, North Chicago, Ill.) as described elsewhere (1), 5 specimens positive for adenovirus, 5 specimens positive for enterovirus, and 5 specimens negative and used as controls.

Antiserum. Rabbit anti-SA11 antiserum was kindly provided by N. Anderson and F. Doane and prepared as described previously by Hopley and Doane (6). The antiserum had a complement fixation titer of 1:3,200 and was inactivated at 55°C for 30 min and ultracentrifuged for 30 min. Dilutions were made in PBS, pH 7.2.

Protein A. Protein A (Pharmacia, Uppsala, Sweden) was dissolved in sterile distilled water to a concentration of 5 mg/ml and frozen in aliquots. For use, this preparation was diluted in PBS, pH 7.2, and used in concentrations from 1 to 200 μg/ml for coating grids.

Colloidal gold antibody. Goat anti-rabbit immunoglobulin G (IgG) labeled with colloidal-gold grains (15 nm in diameter) was purchased from Janssen Pharmaceutical (Piscataway, N.J.); dilutions of the gold-IgG complexes were made in PBS containing 0.1% bovine serum albumin. Each batch of gold-labeled antibody was tested by comparing its labeling of rotavirus with the labeling by an earlier batch of antibody known to label rotavirus.

Electron microscopy. (i) SPIEMDAGT. Formvar-coated grids (300 mesh) were floated for 15 min on 10 μl of protein

A solution and drained. They were then floated sequentially on 10 μl of guinea pig antiserum at room temperature for 20 min, on 20 μl of virus suspension at 37°C for 45 min, on 10 μl of rabbit antirotavirus antiserum at room temperature for 20 min, and on 10 μl of gold-labeled goat anti-rabbit IgG antibody at room temperature for 20 min, with five washes with PBS after each step and then two washes with distilled water before being negatively stained with 1% phosphotungstic acid, pH 7.2, for 5 s.

(ii) DEM. DEM was performed by floating the grids on 20 μl of viral suspension for 3 min; the grids were then negatively stained for 30 s in a 10-μl drop of 1% phosphotungstic acid.

(iii) DIEM. Direct immune electron microscopy (DIEM) was performed as described elsewhere by mixing equal volumes (20 μl) of antigen and antiserum (dilution of 1:500) in a microdilution well (11). Formvar-coated grids were then placed into the wells and incubated for 60 min at 37°C in a humidified environment.

Enzyme immunoassay. The EIA for human rotavirus and SA11 was performed with the Pathfinder enzyme-linked immunosorbent assay kit, as described previously (1). Cutoff values and grey zones (± 10% of the cutoff value) were calculated according to the instructions of the manufacturer. A positive specimen was defined as having a value greater than or equal to the upper grey-zone cutoff value.

Controls and quantitation. The specificity of SPIEMDAGT was assessed by using preimmune guinea pig serum and rabbit antiserum as capture antibody, guinea pig antiserum as detector antibody, and adenovirus-positive, enterovirus-positive or negative stool specimens.

All specimens were examined with a Philips 301 electron microscope at a magnification of ×34,000. For quantitative determination, three grids of each sample were prepared. Virus particles trapped on three grid squares of each of the three grids were counted, and the average number of virus particles per grid square (90 by 1 μm^2) was calculated. Background gold labeling was determined from a control grid containing all reagents except virus and was subtracted from particle counts. Routinely, we consider positive only those stool specimens containing morphologically recognizable rotavirus decorated with gold particles.

RESULTS

Optimal conditions for trapping virus. The optimal concentration of protein A and the optimal capture antibody dilution ratio for trapping virus by SPIEMDAGT were 10 to 50 μg/ml and 1:500, respectively. Protein A concentrations of 10 to 50 μg/ml gave the highest virus counts, with a 5- to 10-fold increase in the number of viruses trapped on grids coated with protein A compared with the number trapped on grids without protein A. Guinea pig capture antibody used at 1:500 gave virus counts about three times higher than a dilution of 1:1,000 and 1.5 to 2 times higher than dilutions of 1:50 and 1:100 (data not shown). The optimal time for coating grids with guinea pig capture antibody was 20 min at room temperature. The highest number of virus particles was achieved with incubation times of 45 min at 37°C and 90 min at room temperature (Fig. 1).

Optimal conditions for viewing trapped virus. Three dilutions of detector antiserum and gold conjugate were used to determine the optimal dilutions of these reagents (Table 1). Although the percentage of virus particles labeled with a 1:5 dilution of gold conjugate was slightly higher than the percentage labeled with a 1:10 dilution, some nonspecific

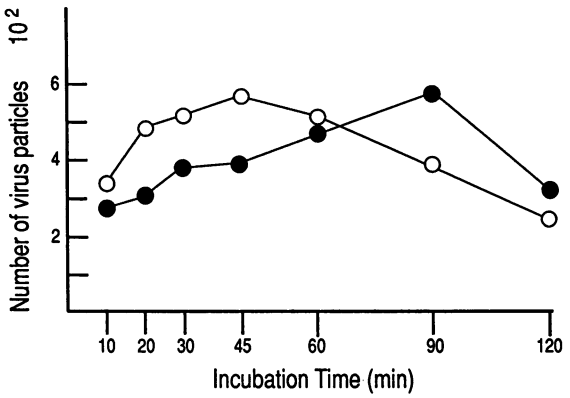

FIG. 1. Time kinetics of virus capture by SPIEMDAGT at 37°C (○) and room temperature (●). Values represent the mean number of particles on six randomly selected grid squares.

adsorption of gold to the grid was observed at the higher conjugate concentration (nonspecific adsorption was defined as the number of gold particles per square micrometer not associated with recognizable virus). In contrast, a 1:20 dilution of gold conjugate failed to label virus efficiently, with about half of the viruses remaining unlabeled. The dilution ratios of virus and detector antibody appeared to have less effect on the extent of virus labeling. Tests of different incubation times with gold conjugate showed that about 50% of virus particles were labeled with gold after 5 min. Incubation times of 10 min resulted in 82% of the virus particles being labeled with gold, while the highest extent of labeling (>99%) was reached at 15 min (data not shown).

Morphology of SPIEMDAGT reaction. Rotavirus SA11 and human rotavirus in fecal specimens were specifically labeled by SPIEMDAGT and included both single-shelled and double-shelled virus particles (Fig. 2A). As would be expected, the majority (ca. 60%) of the rotavirus particles observed by SPIEMDAGT were scattered, while the remainder were seen in groups of two to four particles. Usually, viruses were surrounded with detector antibody molecules, which produced a halo made up to gold particles attached to the detector antibody layer; this led to an increase in the diameter and visibility of the virus (Fig. 2B). This pattern of

TABLE 1. Determination of optimum dilution of gold-IgG complexes with various dilutions of detector antibody

Dilution of gold-IgG complex	No. of gold sol grains labeling each virus particle	% of rotavirus particles labeled with gold grains at detector antibody dilution		
		1:100	1:500	1:1,000
1:5	0	2.4	1.8	5.7
	1–4	63.8	57.2	61.4
	5–9	27.0	34.5	25.3
	>10	6.8	6.5	7.6
1:10	0	5.6	2.1	13.6
	1–4	75.8	71.8	68.5
	5–9	17.3	24.4	16.1
	>10	1.3	1.7	1.9
1:20	0	43.2	52.3	60.7
	1–4	44.8	41.2	36.3
	5–9	9.6	5.2	2.5
	>10	2.4	1.3	0.5

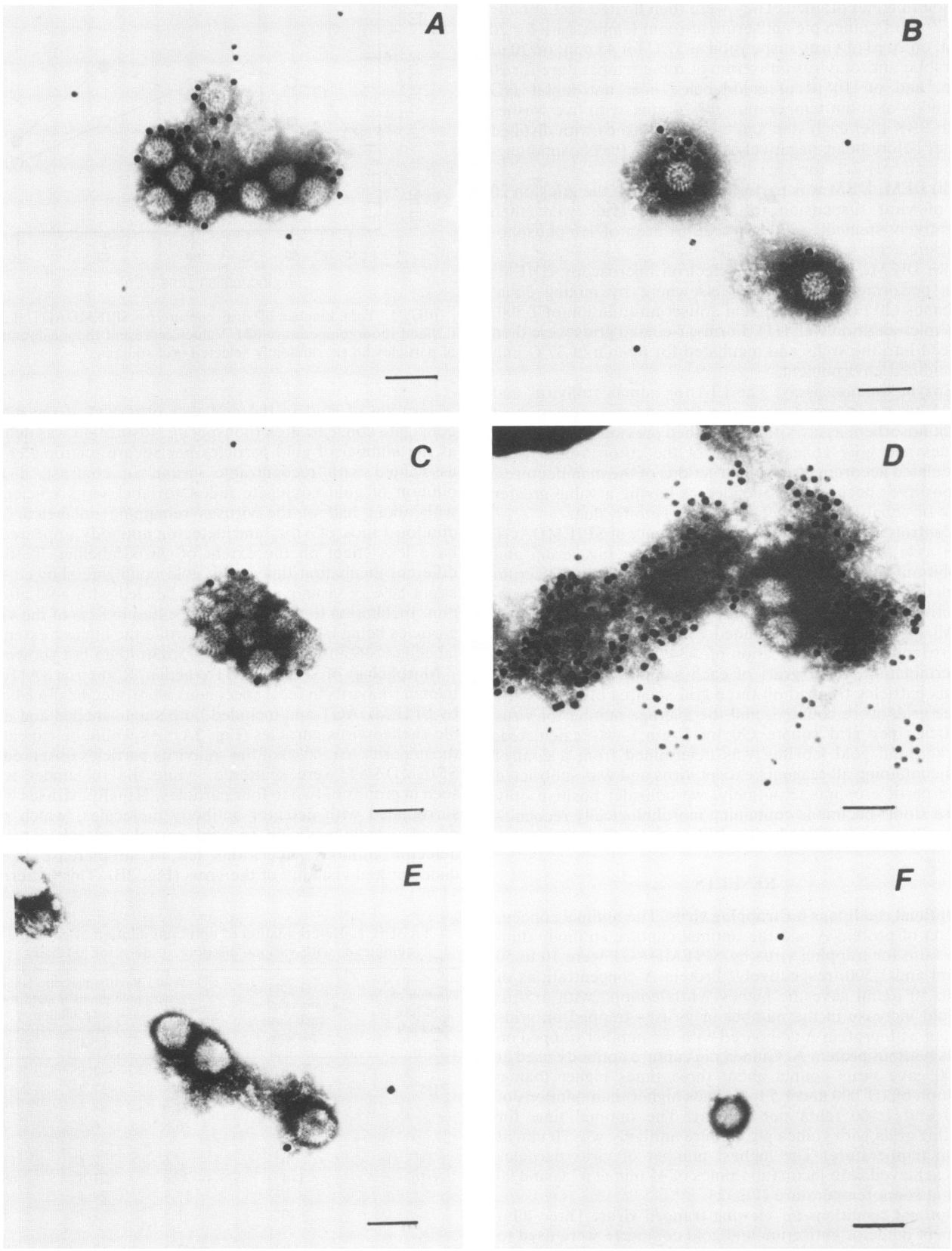

FIG. 2. (A) Rotavirus particles specifically labeled with gold-IgG complexes (diluted 1:10) after guinea pig antiserum as capture antibody (diluted 1:500) and rabbit antiserum as detector antibody (diluted 1:500) were used. (B) Rotavirus in a stool specimen. A detector antibody halo to which colloidal-gold reagent is attached is shown. (C and D) Bizarre forms of rotavirus labeled with colloidal gold. (E) Unlabeled enteric adenovirus. (F) Unlabeled enterovirus. Bars, 100 mm.

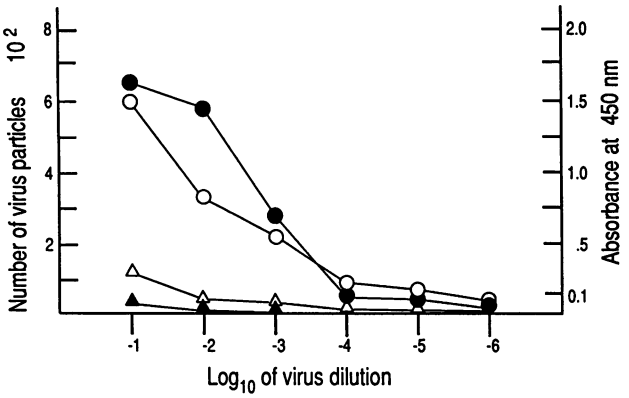

FIG. 3. Comparison of the sensitivities of SPIEMDAGT (○), EIA (●), DIEM (△), and DEM (▲) for the detection of human rotavirus in a stool specimen. Numbers of virus particles are the means of results for six randomly selected grid squares. The cutoff value for EIA was 0.017 absorbance units.

staining facilitated the viewing of virus particles and enabled the detection of viruses present in lower numbers and at lower magnifications. Usually, more than one gold particle was associated with each virus particle; however, gold grains rarely surrounded virus particles completely. The optimal conditions for labeling resulted in about half of the viruses being labeled with one to four gold particles and half with five to nine gold particles (Table 1). The specificity of the technique was shown with adenovirus (Fig. 2E) and enterovirus (Fig. 2F). Occasionally, distorted or incomplete viruses lacking the typical morphology were labeled with gold (Fig. 2C and D), and this labeling could be blocked by inserting a competing detector reagent, confirming that these were rotaviruses.

Control experiments showed that when preimmune serum was substituted for capture antibody, virus was absent and a low level of nonspecific adsorption of gold was observed. When the capture and detector antibodies were reversed, i.e., rabbit antiserum was used as capture antibody or guinea pig antiserum was used as detector antibody, either no gold label was associated with the virus or there was increased nonspecific background staining. Similarly, no gold particles attached to virus were observed when the detector antibody was omitted or replaced by preimmune serum. Control stool specimens with adenovirus, enterovirus, and no virus showed no labeling of these other viruses (Fig. 2E and F).

Comparison of SPIEMDAGT, EIA, DEM, and DIEM methods. The SPIEMDAGT and EIA methods used in these experiments were compared by using known concentrations of serially diluted SA11 rotavirus and a rotavirus-positive stool specimen, all of which had also been examined in parallel by DEM and DIEM. The results for a serially diluted stool specimen assayed by these methods are shown in Fig. 3. For both the tissue culture SA11 virus (data not shown) and the stool specimen (Fig. 3), SPIEMDAGT and EIA could detect virus at higher dilutions than either DEM and DIEM.

The suitability of SPIEMDAGT for testing clinical specimens was evaluated by assaying 20 stool specimens obtained from children with diarrheal disease (Table 2). All 20 specimens were positive by SPIEMDAGT, DEM, and DIEM, but SPIEMDAGT detected many times more virus particles than did the other two electron microscopy techniques. The EIA method, on the other hand, detected only 16 of 20 positive specimens; the 4 EIA-negative specimens had low virus counts by electron microscopy. The SPIEMDAGT method was about 800 times more sensitive than DEM and 45 times more sensitive than DIEM, on the basis of average counts of labeled virus particles.

TABLE 2. Comparison of SPIEMDAGT, DEM, DIEM, and EIA for detecting rotavirus in 20 human stool specimens

Specimen no.	Detection result with:			
	DEM[a]	DIEM[a]	SPIEMDAGT[a]	EIA[b]
1688	2.6	43.0	616.0	+1.276
1055	12.6	149.6	1156.1	+1.154
1668	3.1	16.0	223.5	+1.427
2005	0.6	15.1	245.2	+1.400
1025	1.0	6.3	578.6	+1.284
2319	0.3	5.5	530.4	+1.219
2377	2.0	9.5	791.1	+1.186
1839	2.4	8.5	362.0	Over[c]
1384	39.8	124.0	3935.5	Over[c]
1190	1.5	16.6	155.8	+1.238
1263	24.6	42.0	540.2	Over[c]
1376	20.0	60.0	632.6	Over[c]
1192	8.6	136.0	1082.3	+1.276
1303	46.3	107	662.4	+1.374
1184	8.6	46.3	435.0	+1.264
1382	2.0	13.3	155.0	+1.100
1097	0.3	5.6	69.2	−0.037
963	1.0	11.3	127.2	−0.082
1265	0.5	6.6	88.0	−0.063
1211	1.1	15.0	439.3	−0.019

[a] Average number of rotavirus particles per grid square.
[b] Optical density. The upper grey-zone cutoff value was 0.1413.
[c] Optical density higher than 1.7.

DISCUSSION

We have used SPIEMDAGT to improve the detection of human rotavirus in stool specimens. SPIEMDAGT employs colloidal-gold labeling, which improved the visualization of antigen-antibody reactions by electron microscopy, enabling the detection of both complete rotavirus particles and associated virus antigen present in low concentrations in clinical specimens. SPIEMDAGT was about 800 times more sensitive than DEM and 45 times more sensitive than DIEM.

Others have used SPIEMDAT (SPIEMDAGT without colloidal gold) for the detection of rotavirus (9) and papovavirus (5). In the former study, grids were precoated with protein A to facilitate antibody binding (9), while in the latter study no decorator antibody was employed (5). Papovavirus was detected by using the same antiserum for the capture and decorator antibodies (5). These two methods were reported to increase the sensitivity 3- and 30-fold above the sensitivity of DEM, respectively. Adding gold-IgG complexes to SPIEMDAT has increased the sensitivity of the procedure even further. Our data demonstrate that the use of gold-IgG complexes directed against the detector antibody greatly enhances the visibility of viruses, as rotavirus could be detected at virus dilutions of 10^{-6} (Fig. 3). In addition to the use of protein A and colloidal gold, our study also differed from the previous studies in that we employed two different species of antisera as the capture and detector reagents. In our method, guinea pig antirotavirus antiserum was used as the capture antibody and rabbit antirotavirus antiserum was used as the detector antibody. The use of two

different antibodies greatly reduced nonspecific background staining compared with controls that used rabbit antisera as both capture and detector antibodies, which resulted in high levels of background staining since gold conjugate interacted with both capture antibody and detector antibody.

Optimal conditions for SPIEMDAGT were necessary for obtaining a high degree of specific labeling with minimal background staining. Excess detector antibody did not increase gold labeling of viruses but produced more background staining. Similarly, the concentrations of protein A and capture antibody also influenced the trapping of virus. By SPIEMDAGT, grids coated with protein A plus antibody trapped more particles than did the grids treated with antiserum alone, and the use of optimal concentrations of both protein A and capture antibody contributed significantly to the success of the test.

The SPIEMDAGT specifically labeled rotavirus with a double-antibody layer, which enabled detection of low concentrations of virus at low magnification by increasing the apparent diameter of virus. The optimal conditions for labeling resulted in about half of the viruses being labeled with one to four gold particles and half being labeled with five to nine gold particles. SPIEMDAGT could detect not only morphologically recognizable virus particles but also viral antigens (single or altered double-membrane viruses or capsomeres) in the absence of whole virus particles. Although we would feel uncomfortable diagnosing an infection by seeing gold-labeled disrupted virus or viral antigen in the absence of recognizable virus particles, this observation could suggest that the stool specimen should be reexamined for rotavirus by electron microscopy or be examined by an alternate technique such as EIA, counterimmunoelectrophoresis, or polyacrylamide gel electrophoresis.

ACKNOWLEDGMENTS

We thank S. Lothak, Ernie Spitzer, and Mike Moore for their assistance in electron microscopy; Francis Doane and Nan Anderson for providing us with rotavirus SA11 and antisera; and Santina Castriciano for assistance with EIA.

LITERATURE CITED

1. **Chernesky, M., S. Castriciano, J. Mahony, M. Spiewak, and L. Schaefer.** 1988. Ability of TESTPACK ROTAVIRUS enzyme-immunoassay to diagnose rotavirus gastroenteritis. J. Clin. Microbiol. **26**:2459–2461.
2. **Derrick, K. S.** 1973. Quantitative assay for plant viruses using serologically specific electron microscopy. Virology **56**:652–653.
3. **Doane, F. W., and N. Anderson.** 1977. Electron and immunoelectron microscopic procedures for diagnosis of viral infections, p. 505–539. *In* E. Kurstack and C. Kurstack (ed.), Comparative diagnosis of viral diseases, vol. 2., Academic Press, Inc., New York.
4. **Faulk, W. P., and G. M. Taylor.** 1971. An immunocolloidal method for gold electron microscopy. Immunochemistry **8**: 1081–1083.
5. **Giraldo, G., E. Beth, J. Lee, E. de Harven, and M. Chernesky.** 1982. Solid-phase immune electron microscopy-double-antibody technique for rapid detection of papovaviruses. J. Clin. Microbiol. **15**:517–521.
6. **Hopley, J. F. A., and F. N. Doane.** 1985. Development of a sensitive protein A-gold immune electron microscopy method for detecting viral antigens in fluid specimens. J. Virol. Methods **12**:135–147.
7. **Kjeldsberg, E.** 1985. Specific labelling of human rotavirus and adenoviruses with gold IgG complexes. J. Virol. Methods **12**:47–57.
8. **Milne, R. G., and E. Luisoni.** 1977. Rapid immune electron microscopy of virus preparations. Methods Virol. **6**:265–281.
9. **Nicolaieff, A., G. Obert, and M. H. V. van Regenmortel.** 1980. Detection of rotaviruses by serological trapping on antibody-coated electron microscope grids. J. Clin. Microbiol. **12**:101–104.
10. **Shuala, D. D., and K. H. Gough.** 1979. The use of protein A, from *Staphylococcus aureus* in immune electron microscopy for detecting plant virus particles. J. Gen. Virol. **45**:533–536.
11. **Wu, B., J. B. Mahony, and M. A. Chernesky.** 1989. Comparison of three protein A-gold immune electron microscopy methods for detecting rotaviruses. J. Virol. Methods **25**:109–118.