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Improved Detection of Viruses by Electron Microscopy After Direct Ultracentrifuge Preparation of Specimens

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We have adapted the Beckman Airfuge air turbine ultracentrifuge and the new EM-90 particle-counting rotor to improve detection by electron microscopy of viruses in clinical specimens. Samples were clarified by centrifugation, pelleted in the EM-90 rotor directly to Formvar-coated copper grids, and stained with 1.5% sodium phosphotungstate. Virus counts and endpoint titrations of serial dilutions of partially purified preparations of poliovirus, SA11 rotavirus, herpes simplex virus, and vaccinia virus showed an increase of ca. 1.5 $\log_{10}$ to 3.0 $\log_{10}$ over the virus titers of unconcentrated preparations of the same material. An increased yield of 14% more positive specimens for rotavirus was obtained after preparation of clinical samples by direct ultracentrifugation versus a method without virus concentration (82 versus 72). A prospective study showed that detection of adenoviruses, herpesviruses, and enteroviruses increased when specimens were prepared by direct ultracentrifugation. Direct ultracentrifugation with the EM-90 rotor in the Airfuge ultracentrifuge is a rapid concentration method which enhances the rate and yield of virus detection from clinical specimens by electron microscopy and is easily adaptable to a diagnostic virology laboratory.

Electron microscopes have proved to be very useful for the detection of a wide variety of viruses in clinical specimens (5, 8, 11, 13). Rapid identification of viruses into groups can be made on the basis of virus morphology (6).

The objective of this study was to test the hypothesis that direct ultracentrifugation of viruses to the specimen grid, instead of the use of the unconcentrated preparation method, would increase detection of viruses in clinical specimens when examined by the electron microscope. To evaluate this hypothesis, we used the newly designed Beckman EM-90 particle-counting rotor with the Beckman Airfuge air turbine ultracentrifuge for direct pelleting of virus to the specimen grid, followed by negative staining and examination by electron microscopy (EM). We found this technology to be sensitive and applicable to routine use with clinical specimens submitted to a diagnostic virology laboratory.

MATERIALS AND METHODS

To examine the principle of this study by mathematical analysis, we calculated the theoretical increase in the relative concentration of virus particles after they were ultracentrifuged in the EM-90 rotor. We calculated this by estimating the post-ultracentrifugation reduction in volume of distribution of viruses from their original 100-μl suspension volume in the EM-90 rotor. This approach assumes that all viruses are pelleted by ultracentrifugation (see calculations below). Calculating the increase in the relative concentration of viruses also assumes that viruses are distributed randomly. This assumption is based upon the experience of Miller and Sharp (16). Trapezoid-shaped centrifuge cells whose walls have radii corresponding to the radius of the rotor, such as the cells found in the EM-90 rotor, produce minimal convection, which results in even particle distribution for particle counting accuracy (17, 20, 21).

To calculate the relative concentration after direct ultracentrifugation, we assumed that the volume of distribution of the ultracentrifuged viruses was the area of the rotor cell base (25 mm^2) times the height, i.e., the diameter of a pelleted virus (estimated for purposes of this calculation to be 100 nm).

The theoretical relative concentration factor of ultracentrifuged virus particles = the initial volume of virus distribution/the final volume of virus distribution = 100 $mm^3/25$ $mm^2 \times$ virus diameter (ca. 100 nm) = 100 $mm^3/25 \times 10^{-4}$ $mm^3 = 4 \times 10^4$. Therefore, the approximate concentration factor of the EM-90 rotor used with air turbine ultracentrifugation is $10^4\times$ for particles the size of many human viruses, over any area of the rotor cell base.

To evaluate whether this theoretical concentration factor improved the sensitivity of virus detection in actual laboratory practice, we set up the following experiments for detection of viruses of various sizes in nonclinical specimens and clinical specimens submitted to a diagnostic virology laboratory.

Nonclinical specimens. The time (t) required to pellet virus particles can be predicted by calculations based on the gravitational force generated during ultracentrifugation, the duration of centrifugation, and

the virus sedimentation coefficient. This calculation is expressed in the formula $t = k/s$ (3), where k equals the pelleting efficiency factor and s equals the particle sedimentation coefficient in Svedberg units (S). The EM-90 rotor is designed to operate at a maximum of 30 lb/in^2 air pressure, providing a run speed of 90,000 rpm, with k equal to 19 (4). Because our on-line air pressure generated only 26 lb/in^2 after filtration, it was necessary to calculate k at 85,000 rpm (see Fig. 2; [4]). From the formula k = (maximum run speed/actual run speed)^2 × k maximum run speed (3): k (85,000 rpm) = (90,000/85,000)^2 × 19 = 21.3.

From this value of k, we calculated the time (t) to pellet a particle having the characteristics of the 20-nm hepatitis B surface antigen particle, 45S (10): we predicted that t = 21.3/45 = 0.47 h = 28.2 min. Thus, a 30-min ultracentrifugation would pellet small, low-density subvirus particles in the EM-90 rotor under the above conditions. Parvoviruses, which are very small, have sedimentation coefficients of 110 to 122S (14) and would require even less centrifugation time.

The following nonclinical virus specimens were used for comparison of virus quantitation after direct ultracentrifugation with a method which used unconcentrated material. (i) The seed stock of the SA11 strain of rotavirus was obtained from the supply of M. Gurwith, Department of Medical Microbiology, University of Manitoba. Virus was harvested at a 4+ cytopathic effect from SA11-infected primary African green monkey kidney tissue culture cells (Connaught Laboratories, Inc., Willowdale, Ontario), and the cells were frozen and thawed three times. Virus in the supernatant fluid was separated from the cell pellet after preliminary centrifugation at 1,000 × g for 15 min. A purified virus suspension was obtained after the virus was pelleted from the supernatant by ultracentrifugation in a Beckman type 35 rotor at 82,000 × g for 1 h, followed by suspension of the pellet in phosphate-buffered saline, and then rate zonal ultracentrifugation in a Beckman SW27.1 rotor at 83,000 × g for 1 h. Two successive sucrose gradients were used: 12, 24, 36, 48, and 60% (wt/vol) sucrose in 0.1 M tris(hydroxymethyl)aminomethane buffer (pH 7.5). The virus band, detected by negative staining with 1.5% sodium phosphotungstate (pH 7.0), was pelleted in a Beckman type 50 rotor at 145,000 × g for 1 h and suspended in phosphate-buffered saline (pH 7.2). The hemagglutination titer of the purified SA11 rotavirus suspension tested with human type O erythrocytes was 512 hemagglutination units per 0.5 ml. (ii) Vaccinia virus vaccine was obtained from Connaught Laboratories, Inc. and was reconstituted from the lyophilized state to vaccine strength. (iii) Herpes simplex virus was grown from a genital isolate of herpes simplex virus-infected primary human amnion cells which were harvested at a 4+ cytopathic effect, frozen and thawed three times, and clarified by centrifugation at 1,000 × g. (iv) Poliovirus type 1 was obtained from the clarified supernatant of infected primary African green monkey kidney cells (Connaught Laboratories, Inc.), harvested at a 4+ cytopathic effect, and frozen and thawed three times.

These specimens were divided into portions and prepared by two methods for EM examination. The unconcentrated method, a modification of the technique of Almeida (2) called the rapid procedure, involved applying the virus suspension to a Parafilm square and then floating a Formvar-coated 400-mesh copper grid, Formvar side down, on this drop of virus suspension. After 1 min the grid was removed, the excess suspension was absorbed with the ragged edge of a torn piece of filter paper, and 1.5% sodium phosphotungstate stain (pH 7.0) was added to the grid. The ultracentrifugation method, called the EM-90 preparation, utilized a filter paper square measuring 5 by 5 mm with a 400-mesh copper grid attached directly to the filter paper by a Formvar coat and placed in the EM-90 rotor cell base. After sonication for 60 sec at 30% maximum power on an Artek 300 Sonic Dismembrator, 95 μl of each virus suspension was then placed into a rotor cell. Ultracentrifugation was performed at 26 lb/in^2 air pressure for 30 min. The specimen grid was removed, the supernatant was absorbed with filter paper, and 1.5% sodium phosphotungstate stain (pH 7.0) was applied. Excess stain was removed by filter paper absorption, and the grid was allowed to dry. Specimen grids were examined with a Philips 201 electron microscope at 60 kV. All virus counts were obtained by counting with a hand recorder during inspection of at least five grid squares. These grid squares were selected at random from the edge of the four grid quadrants and the central area of the grid.

To confirm the relative virus quantitation observed at a single dilution of these specimens, endpoint dilutions of the virus suspensions were determined from the highest virus dilution in which readily detectable virus particles could be quantitated. Serial $\log_{10}$ or twofold dilutions were made from the original virus suspension in distilled water, and specimens were prepared and examined as described above.

Examination of clinical specimens. For fecal clinical specimens submitted to the Virus Isolation Laboratory of The Cadham Provincial Laboratory, Winnipeg, we employed standard techniques to treat the samples and inoculate them into appropriate tissue culture cell lines. The 10% suspension in Hanks balanced salt solution (pH 7.2) from this original material was used for tissue culture inoculation; it was diluted to a final 2 to 5% suspension divided into two samples, one prepared by the previously described rapid procedure and the other by the EM-90 preparation, before examination with the electron microscope. The sample from the suspension of clinical specimens prepared for direct ultracentrifugation was first sonicated to free clumped viruses, especially rotaviruses (18) (which are stable to sonication [7]) that might pellet during the clarifying centrifugation. (We currently omit this step, since clumping often persists.) Before direct ultracentrifugation, the specimens were clarified with an Eppendorf microcentrifuge, model 5142, at 12,800 × g for 5 min. Specimens were prepared and coded by one individual and examined with the electron microscope over five grid squares by a second individual. The EM-90 rotor was cleaned by immersion of all the sector core components in 2% glutaraldehyde for 30 min followed by brushing with a cotton-tipped swab between specimen preparations.

Sixty-two stored fecal specimens previously positive

for rotavirus, as determined by EM examination with the rapid procedure during routine examination of fecal specimens during the winter of 1979–1980, were reexamined after the rapid procedure and the EM-90 preparation. Further tests of the relative sensitivities of the rapid procedure and the direct ultracentrifugation preparation were made from specimens suspected to contain rotavirus. These included 93 fecal specimens (stored at −70°C) collected from infants less than 3 years old who had gastroenteritis during the winter of 1979–1980. These children had an illness clinically compatible with rotavirus infection, but EM examination of the specimens after they had been prepared by the rapid procedure showed the specimens to be negative for rotavirus. For the current study, samples of the suspensions from these specimens were retested by both the rapid procedure and the EM-90 preparation.

A prospective comparison of the sensitivity of virus detection after the rapid procedure with the sensitivity after the EM-90 preparation was carried out over an 8-week period during the summer of 1980. A total of 176 stool specimens, 46 urine specimens, 7 cerebrospinal fluid specimens, and 6 vesicle specimens were examined by the two EM preparation techniques, and the results were compared with the results of virus cultivation in tissue cultures. An additional 12 specimens, 1 vesicle aspirate positive by culture for varicella-zoster virus and 11 swab specimens positive by culture for herpes simplex virus, were selected at random from clinical samples, placed in 2 ml of virus transport medium, and stored at −70°C, to provide further experience with direct ultracentrifugation before EM. All visualized viruses were identified from characteristic morphology and size measurements determined from photographs taken during EM examination. Enteroviruses were typed according to neutralization by utilizing Lim Benyesh-Melnick antiserum pools (National Institute of Allergy and Infectious Diseases, Bethesda, Md.). Herpesviruses were distinguished by characteristic cytopathic appearances and the time they required for growth on primary human amnion cells, African green monkey kidney cells (Flow Laboratories, Inc., Rockville, Md.), and MRC-5 tissue culture cells (Flow Laboratories, Inc.). Virus particle counts were possible on 17 clinical specimens positive by culture for herpesviruses. Virus infectivity titration for cytomegalovirus (CMV) from the unconcentrated urine of a newborn with severe congenital CMV infection was performed in MRC-5 monolayers (Flow Laboratories, Inc.), four tubes per dilution, from 10^{-6} to 10^{-1} dilutions, which were examined three times per week. The titer was determined at three weeks by the method of Reed-Muench.

All EM photographs to determine if specimens were positive for viruses were made on Kodak fine-grain positive film 5302 (Canadian Kodak Co., Toronto, Ontario).

RESULTS

The results of the particle counting of nonclinical specimens are seen in Table 1. These results show that ca. >1 $\log_{10}$ to >3 $\log_{10}$ more virus particles were seen after direct ultracentrifugation than were seen after the rapid procedure. This relative increase was confirmed by the endpoint titer, which also demonstrated an increased sensitivity of virus detection after the EM-90 preparation of ca. 1.5 $\log_{10}$ to >3.0 $\log_{10}$.

TABLE 1. *Comparison of virus quantitation of nonclinical specimens*

Virus	No. of virus particles[a]		Endpoint titration	
	Rapid procedure	EM-90 prepn	Rapid procedure	EM-90 prepn
SA11 rotavirus	0.8	45.4	1:1,000 (0.8)[b]	1:40,000 (0.4)
Herpes simplex	13.6	>1,000	1:100 (1.6)	1:100,000 (1.0)
Vaccinia	0.2	542.6	1:1,000 (0.2)	1:100,000 (2.8)
Poliovirus	0.75	26.2		

[a] Virus counts per 400-mesh grid square were averaged over five or more grid squares.

[b] Parentheses indicate the average number of virus particles per 400-mesh grid square at the indicated dilution.

Representative appearances of an EM field of a grid for four different viruses, comparing the rapid procedure- with the direct ultracentrifugation-prepared specimens, can be seen in Fig. 1 and 2. These photographs show the marked concentration effect of direct ultracentrifugation on virus distribution.

The results of rotavirus detection are shown in Table 2. Differences between rotavirus detection by the rapid procedure and the direct ultracentrifugation technique were reconfirmed in 9 of 11 patients by repreparation and reexamination of the specimens by both techniques. A total of 55 of the 62 previously positive specimens were reidentified by both the rapid procedure and the EM-90 preparation (Table 2). Neither technique could enable rotavirus to be identified in four specimens. In no specimens was rotavirus detected by the rapid procedure and not by the EM-90 preparation. However, there were four specimens in which direct ultracentrifugation permitted detection of rotavirus but the rapid procedure did not. Reexamination of suspect specimens showed that both the rapid procedure and the EM-90 preparation detected seven rotavirus infections. In none of these specimens was rotavirus detected by the rapid procedure alone. However, an additional six detections of rotavirus were made from this group after the direct ultracentrifugation procedure. The results of both methods for EM examination of rotavirus, determined from a prospective study of fecal specimens during the summer of 1980, are incorporated into Table 2. A total of 10 additional detections of rotavirus were made by EM after direct ultracentrifugation (an increased yield of 14% [10/72]) that would not

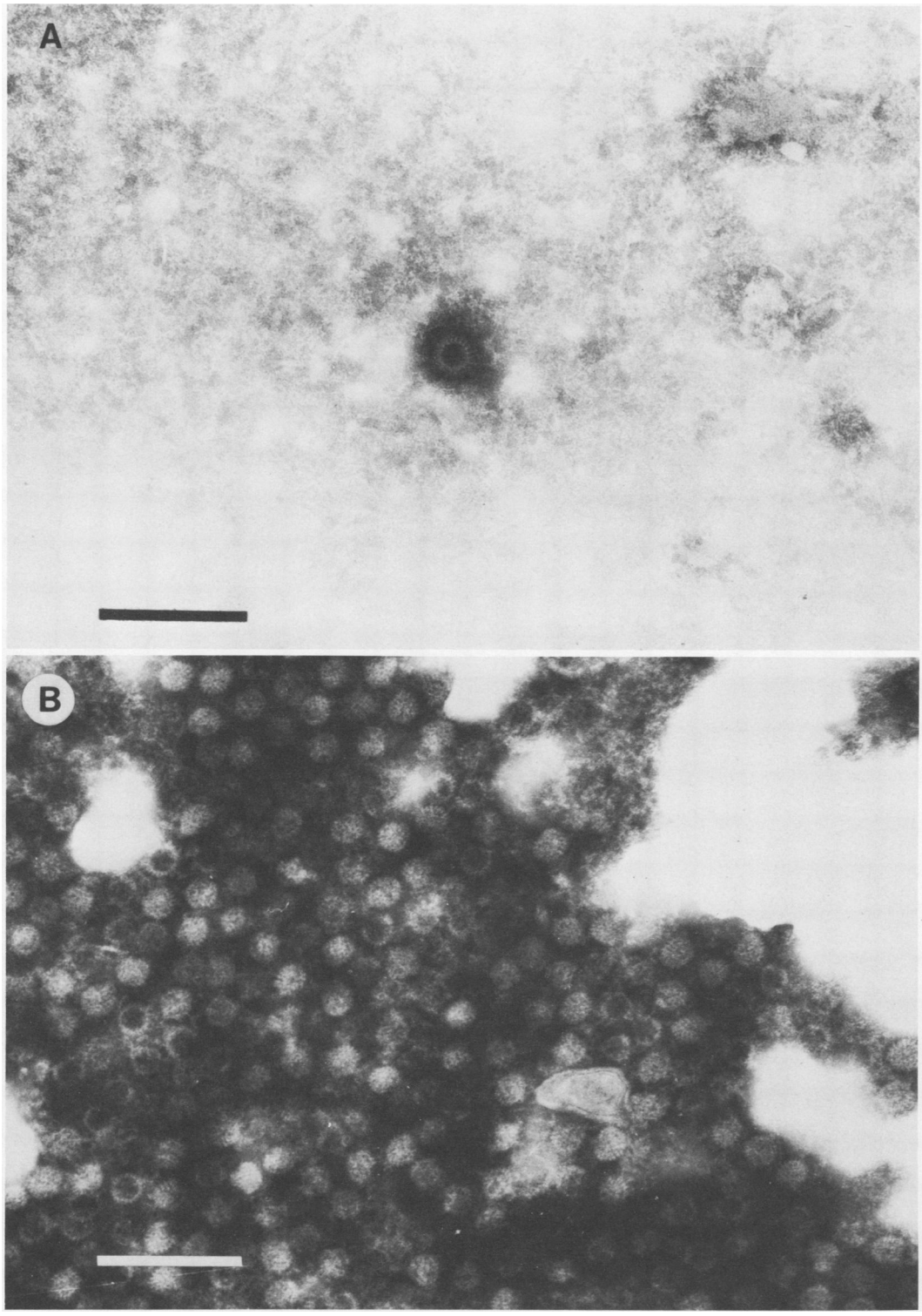

FIG. 1. *EM photographs of representative fields of unconcentrated preparations (A and C) and direct ultracentrifuge preparations (B and D) of two nonclinical specimens containing SA11 rotavirus (A and B) and vaccinia virus (C and D). Bars, 250 nm.*

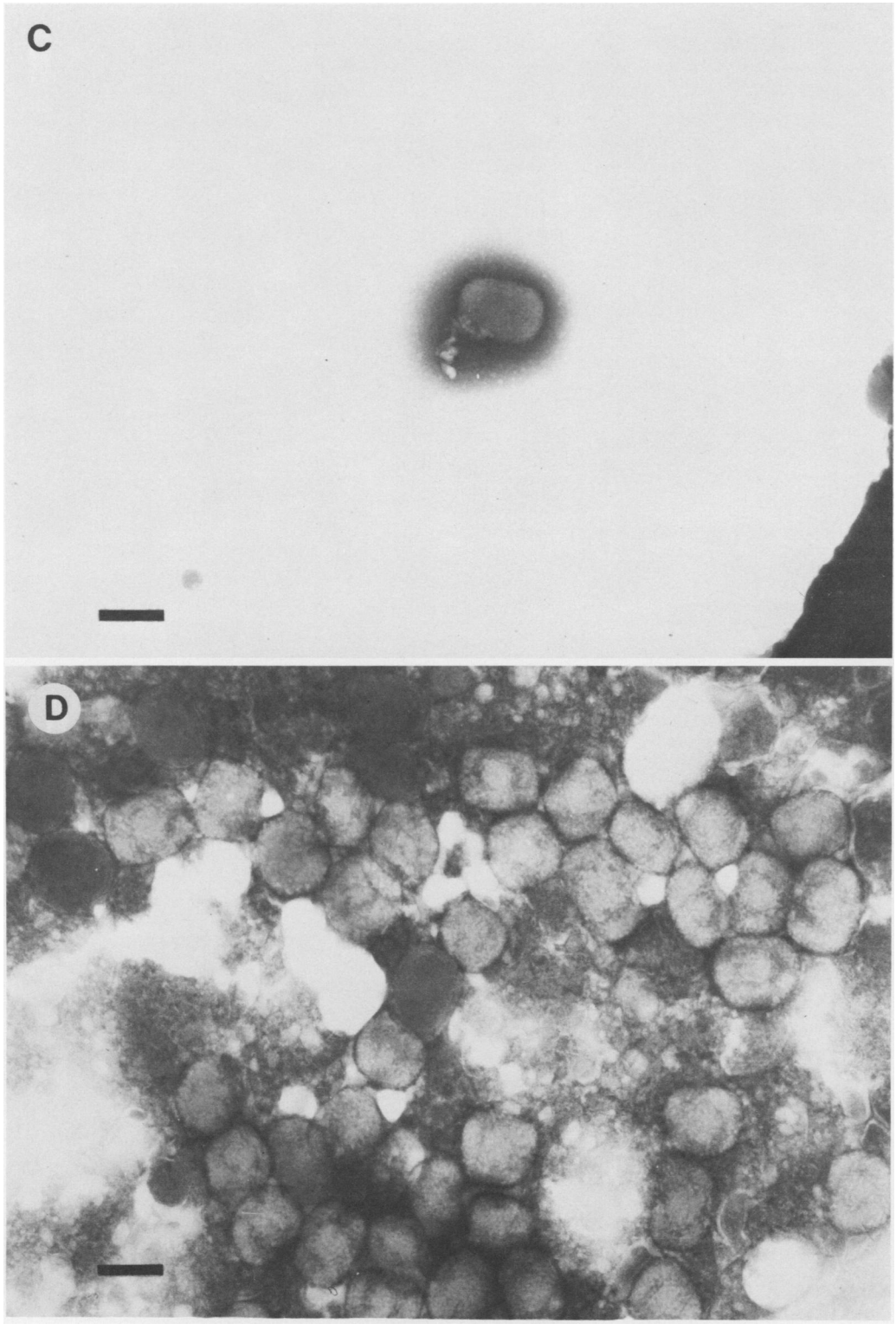

FIG. 1 C and D

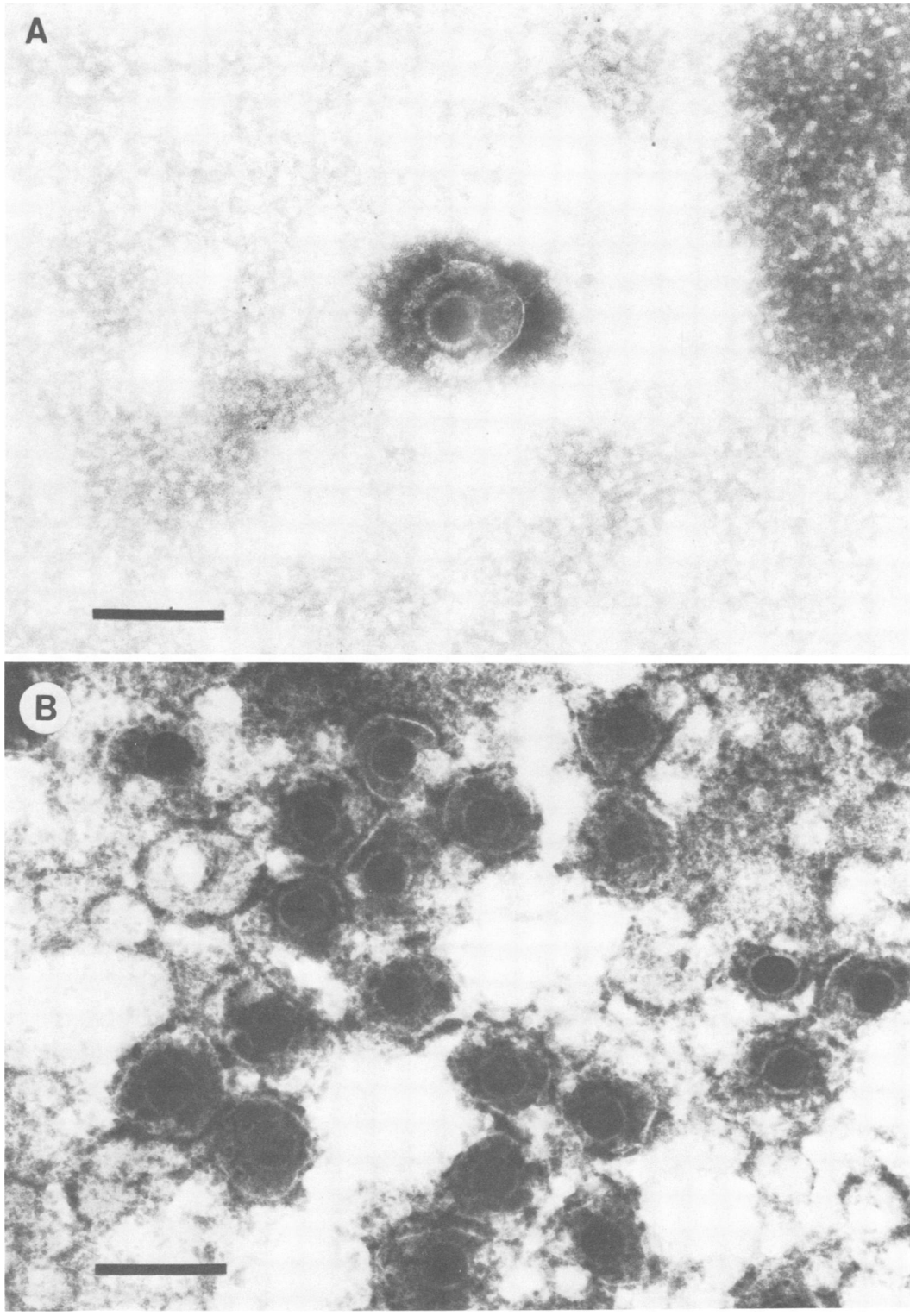

FIG. 2. *EM photographs of representative fields of unconcentrated (A and C) and direct ultracentrifuge preparations (B and D) of two clinical specimens containing varicella-zoster virus from a swab of a skin vesicle (A and B) and human rotavirus from a fecal specimen (C and D). Bars, 250 nm.*

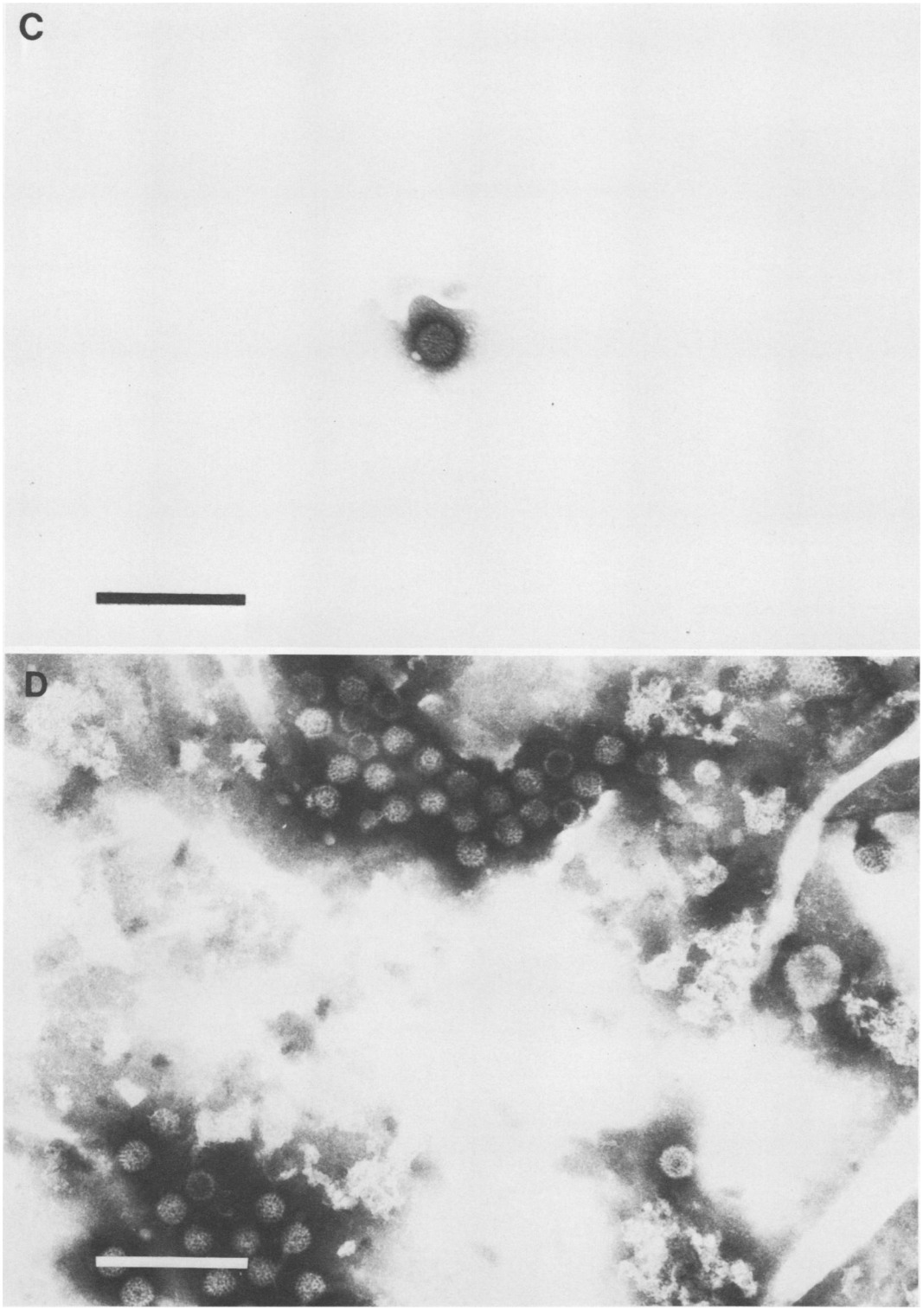

FIG. 2 C and D

TABLE 2. *Comparison of rotavirus detection*

Origin of specimen	No. rapid procedure-positive, EM-90 prepn-negative	No. EM-90 prepn-positive, rapid procedure-negative	No. both EM prepn positive	No. both EM prepn negative
Previously positive specimens (62)	0	3[a]	55	4
New detections from suspect specimens (93)	0	6[a]	7	80
Prospective study specimens (176)	1[b]	1[b]	9	165

[a] The detection of rotavirus was confirmed by repeat specimen preparations and EM examination.
[b] Insufficient specimen available for repeat examination.

have been made after the rapid procedure alone.

In a prospective study of fecal specimens during the summer of 1980, the direct ultracentrifugation technique permitted the detection of an additional 3 specimens positive for adenovirus, 12 positive for entero-like viruses, 1 positive for reovirus, and 1 positive for rotavirus. These 17 positive specimens showed almost a doubling of the yield of viruses visualized by EM after the rapid technique (2 + 19 = 21) (Table 3). The identifications of the enterovirus-positive specimens are shown in Table 4. Of the 176 specimens cultured, enteroviruses were isolated from 29. Three of these isolates were seen after both the EM-90 preparation and the rapid procedure. An additional seven were detected after the EM-90 preparation alone. However, almost two-thirds (19/29) were not detectable after either the EM-90 preparation or the rapid procedure. An additional five entero-like virus detections were made after direct ultracentrifugation alone, but enteroviruses were not cultivated. These entero-like viruses may represent noncultivable enteroviruses on tissue culture, such as group A coxsackieviruses, hepatitis A virus, Norwalk virus, or other small round viruses associated with gastroenteritis. Alternatively, they may represent false-positive detections of entero-like virus particles after the EM-90 preparation which are artifacts. One entero-like virus was detected after both the EM-90 preparation and the rapid procedure, but no virus was cultivated. The extra detection of one entero-like virus and one rotavirus after the rapid procedure may reflect differences in staining results, excess debris in the concentrated specimen, or observer error. No repeat specimen preparations or EM examinations were made for the EM-positive entero-like viruses that were not cultured. Immune EM with acute- and convalescent-phase sera would be one method to confirm the viral nature of these noncultivable, entero-like virus particles, but no serum was obtained from patients in this study.

Of 46 urine specimens, 5 were positive for CMV, and no other viruses were cultivated. From three of these five specimens, herpesvirus particles were seen after the direct ultracentrifugation preparation (Table 5), but only one herpesvirus particle from a congenital CMV-infected infant was visualized after the rapid procedure. These three specimens, found to be positive by EM after the EM-90 preparation, were from two renal transplant patients and one congenital CMV-infected infant. The CMV titer in the urine of the infected infant was 10^4 infectious particles per ml.

Quantitative counts were attempted on 15 vesicle and 6 urine samples, which were positive by culture for viruses of the herpesvirus family (Table 5). In 14 of 15 vesicle specimens positive by culture for herpesviruses and in 3 of 6 urine specimens positive for CMV (including two renal transplant patients), herpesvirus could be visualized by EM after direct ultracentrifugation. Direct comparisons of quantitative counts were possible for three herpes simplex virus culture-positive specimens and two varicella-zoster virus culture-positive specimens, which showed an increase of ca. 2 $\log_{10}$ to >3 $\log_{10}$ in the number of virus particles seen after direct ultracentrifugation.

All cerebrospinal fluid specimens were negative by culture and EM.

DISCUSSION

Ultracentrifugation has been a recognized method for obtaining accurate particle counts of virus suspensions (16, 20). Sharp pioneered procedures which resulted in the development of particle-counting rotors, such as the Sorvall type SU rotor (21). This rotor assembly was rather cumbersome, and the ultracentrifuge could generate only 31,500 × *g*.

The development of the EM-90 rotor by the Spinco Division of Beckman Corporation, Inc. in collaboration with Miller has advanced the technology for counting virus particles (17). We have adapted this air turbine ultracentrifugation system for direct pelleting of viruses from clinical specimens. An estimated theoretical increased concentration for virus particles of ca.

TABLE 3. *Viruses identified from prospective study of 176 fecal specimens*

Virus	No. rapid procedure-positive, EM-90 prepn-negative	No. EM-90 prepn-positive, rapid procedure-negative	No. both EM prepn-positive	Totals		
				Positive by culture		No. culture- and EM-positive[a]
				No. both EM prepn-negative	No. positive cultures alone	
Adenovirus	0	3	5	3	8	11
Entero-like virus	1	12	4	19	29	36
Reovirus	0	1	1	0	0	2
Rotavirus	1	1	9	0	0	11

[a] Four patients had mixed viral infections.

TABLE 4. *Enterovirus isolates detected in fecal specimens*[a]

Virus	No. rapid procedure-positive, EM-90 prepn-negative	No. EM-90 prepn-positive, rapid procedure-negative	No. both EM prepn-positive	No. both EM prepn-negative
Poliovirus 1	—[b]	—	—	3
Poliovirus 2	—	2	2	5
Poliovirus 3	—	2	—	2
Polioviruses 1 and 3	—	1	—	—
Polioviruses 2 and 3	—	1	—	—
Echovirus 7	—	1	—	2
Echovirus 24	—	—	1	1
Coxsackie A9	—	—	—	2
Coxsackie B2	—	—	—	1
Coxsackie B4	—	—	—	1
Unidentified	—	—	—	2
Virus cultured	0	7	3	19
No virus cultured	1	5	1	0

[a] Total specimens in which virus was cultured, 29. Total specimens in which no virus was cultured but in which virus was visualized by EM, 7.

[b] —, Not detected.

TABLE 5. *Herpesviruses visualized in clinical specimens by EM*

Virus and source	No. rapid procedure-positive, EM-90 prepn-negative	No. EM-90 prepn-positive, rapid procedure-negative	No. both EM prepn-positive	No. both EM prepn-negative
Herpes simplex				
Swab (11)[a]	—[b]	8 (0;4.8)[c]	2 (0.3;42.2)	1
Aspirate (1)	—		1 (3.4;>9,000)	
Varicella-zoster				
Swab (1)	—		1 (0.2;119.6)	—
Aspirate (2)	—	1 (0;5.6)	1 (15;>2,000)	—
CMV				
Urine (6)	—	2 (0;6.8)	1 (0.2;ND[d])	3

[a] Numbers in parentheses after virus sources indicate numbers of specimens.

[b] —, No virus visualized.

[c] The first number in each set of parentheses represents the rapid procedure virus counts per 400-mesh grid square averaged from five or more grid squares; the second number represents the EM-90 preparation virus counts for the number of specimens indicated.

[d] ND, Not determined.

10^4 which would result from the use of this technology led us to evaluate EM detection of viruses in specimens after direct ultracentrifugation.

The virus particle counts of a variety of viruses, ranging from enteroviruses to poxviruses,

have shown an increase of ca. 1.5 $\log_{10}$ to 3.0 $\log_{10}$ in particle counts after direct ultracentrifugation. This was verified by comparison of the endpoint titers at which nearly equivalent particle quantitations were made. Thus, for these relatively purified virus suspensions, direct ultracentrifugation produced an approximately 50- to >1,000-fold increase in the sensitivity of virus particle detection by EM.

However, it was necessary to conduct a study of clinical specimens which contain debris that may interfere with EM (2) after direct ultracentrifugation. Clarification of clinical specimens for five min with the Eppendorf microcentrifuge, model 5412 (which generates ca. 12,800 × *g*), was a rapid method to reduce the background debris preceding ultracentrifugation in the EM-90 rotor. Careful removal of the supernatant after this clarification step was essential to ensure that the pellet was not disturbed before this supernatant was placed in the EM-90 rotor cells. Also, fecal suspensions were made to a 2 to 5% concentration to minimize debris which might pellet during ultracentrifugation. To avoid cell-to-cell contamination in the EM-90 rotor during ultracentrifugation, the cells were not overfilled; we used a 95-μl volume of sample, and the rotor cover was tightly closed after the gasket was lightly greased for each run.

The increased sensitivity of virus detection which resulted from direct ultracentrifugation was useful in increasing the yield of virus-positive specimens detected by EM examination both in the retrospective examinations for rotavirus (14% [10/72] additional detections of rotavirus after direct ultracentrifugation that would have been missed after the rapid procedure alone) and in the prospective study of viruses in fecal, urine, and vesicle samples. The reason that we failed to reconfirm rotavirus detections in seven of the previously positive fecal specimens by the rapid procedure could be attributed to several factors. The differences between the original rapid procedure carried out in the winter of 1979–1980 and the repeat rapid procedure included the following: a different stool suspension of higher concentration was used in the earlier examination; the original rapid procedure followed centrifugation of the stool suspension at 10,000 rpm for 30 min, whereas the repeat rapid procedure was unclarified; the specimens were frozen and thawed many times before and during this study; and different observers performed the EM examinations. The increased yield of rotavirus detections after direct ultracentrifugation in the EM-90 rotor may have been due to the increased ease of rotavirus detection at the height of virus shedding or to the increased sensitivity of this technique when titers of excreted virus diminished with convalescence. Our experience has been that when virus is detected by EM after direct ultracentrifugation, virus particles are readily visualized in the first or second grid square.

In the prospective study, the versatility of the EM-90 preparation in detecting a wide range of viruses was shown by the increased detection of adenoviruses, entero-like viruses, reoviruses, and herpesviruses, although the number of positive specimens was small. We considered the entero-like virus particles to be present only after we examined photographs which confirmed shape and size. The difficulty in detection of enteroviruses by EM reflects the lack of definitive morphology, the small size, and the low fecal excretion titers of these viruses. (1, 15).

Diagnostic virology laboratories have evaluated the number of infectious particles excreted in human fecal specimens after enterovirus infections (15). The laboratories have reported titers of poliovirus (range of infectivity, 3.0 $\log_{10}$ to 6.5 $\log_{10}$; 50% tissue culture infective dose per gram of stool) higher than those of coxsackieviruses and echoviruses (2.0 $\log_{10}$ to 5.5 $\log_0$; 50% tissue culture infective dose). A further elevation of titers was found when more sensitive human cell culture lines were used, such as human kidney cells used for poliovirus detection (15). Thus, it would not be surprising that some enteroviruses, especially polioviruses, would be visualized by sensitive EM, although many excreted enteroviruses would be expected to be below the threshold for EM detection and would be detected only by cultivation. Although our detection of enteroviruses by EM was limited, the enteroviruses most frequently visualized after direct ultracentrifugation were the polioviruses. Overall, however, approximately one-third (10/29) of the cultivated enteroviruses were seen by EM (Table 4).

The feasibility of detecting CMV by EM after the use of the pseudoreplica specimen preparation method has been demonstrated for congenital CMV infections, especially when the urine titer of virus frequently exceeds 10^4 infectious particles per ml (13). After renal transplantation, direct ultracentrifugation may assist the rapid diagnosis of some cases of acquired or reactivated CMV infections (two of five in our study).

Immune EM can be ca. 100 times as sensitive as standard negative staining EM examination (6, 12). Direct ultracentrifugation offers an increase in sensitivity similar to or greater than that of immune EM and has several attractive advantages. The direct ultracentrifugation procedure described here is more rapid, requires no serum incubation step, and does not require a range of specific immune sera.

The technology for direct ultracentrifugation utilized in this study includes several features important for a diagnostic laboratory. Because the rotor is driven by air pressure and has no mechanical parts to produce friction, no vacuum or refrigeration system is required. Thus, the Airfuge ultracentrifuge with the EM-90 rotor is compact, has reduced run time due to rapid acceleration and deceleration, is less expensive, and uses a minimum of electrical power, as compared with a conventional ultracentrifuge. This technology is simple, rapid, and safe and involves a minimum of preparative time on the part of the technologist. The additional advantage of direct ultracentrifugation over conventional ultracentrifugation in preparing specimens for EM detection of viruses is that less virus is lost or diluted when the ultracentrifuged pellet is resuspended.

A recent study has demonstrated an additional 6.5% (3/46) increase in rotavirus infection detection by EM after specimen preparation by conventional ultracentrifugation, as compared with clarification of fecal specimens alone (19). Other types of viruses from fecal specimens were detected at an equal rate after both specimen preparation methods. The conclusion of that study was that ultracentrifugation was not a worthwhile preparative step for the minimal increase in yield. However, the use of the new technology described here for direct ultracentrifugation avoids the difficulties which were encountered with conventional ultracentrifugation (19). Also, direct ultracentrifugation has potentially greater sensitivity than indirect ultracentrifugation, because the virus recovery step is eliminated.

Gelderblom et al. have recently reported the use of a special adapter in a rotor model earlier than the EM-90 which allowed direct ultracentrifugation in the Airfuge ultracentrifuge (9). They noted a >100-fold-increased detectability of adenovirus grown in cell culture and also an increased sensitivity of virus detection from clinical specimens.

We have integrated the use of the Airfuge ultracentrifuge with the EM-90 rotor into our diagnostic virology laboratory and are currently processing all specimens submitted for EM examination by direct ultracentrifugation.

ACKNOWLEDGMENT

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ADDENDUM IN PROOF

We have performed a preliminary evaluation of a newly designed sector insert for the EM-90 rotor in which the EM specimen grid can be positioned directly in the sector cell without a filter paper support. We found that the new sector insert further simplifies this direct ultracentrifugation technique.

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