Journal of Medical Virology 29:192–195 (1989)

Electron Microscopy of Stool-Shed Viruses: Retention of Characteristic Morphologies After Long-Term Storage at Ultralow Temperatures

Fred P. Williams, Jr.

Virology Branch, Microbiology Research Division, Environmental Monitoring Systems Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio

Storage of stool specimens at −70°C has been reported to destroy the characteristic morphology of calicivirus. To determine if other stool-shed viruses are similarly affected, stool specimens previously examined by electron microscopy and observed to contain virus particles were reexamined after 6–10 years of storage at −70°C to −85°C. The stools contained virus particles of different morphological types, including astrovirus, small round structured virus, adenovirus, and rotavirus as well as calicivirus. Also reexamined were stools containing coronavirus-like particles and T = 19 virus-like particles. Characteristic virus particles, including calicivirus particles, were recognized in all the stools reexamined. The results indicate that long-term storage of stools at ultralow temperatures does not present a significant problem for the morphological identification of stool-shed viruses.

KEY WORDS: astrovirus, calicivirus, SRSV, adenovirus, rotavirus, virus-like particles

INTRODUCTION

Unlike other methods used to examine stools for viruses, electron microscopy (EM) permits the visualization and morphological characterization of individual virus particles. With EM, a stool specimen can be scanned for a number of morphologically distinct viruses in a single examination. EM does not require cell culture and is not confined, as are other methods, to detecting viruses with common antigens or similar genomes. Specimens must, however, be examined one at a time with EM. This can be time-consuming if many specimens are to be examined. When large numbers of specimens are involved, as frequently happens during outbreaks of illness, proper storage of the specimens is essential.

Commonly, specimens awaiting viral analyses are stored at −70°C [Lennette et al., 1980]. However, it has been reported that such frozen storage destroys the characteristic morphology of at least one virus found in stools, calicivirus [Humphrey et al., 1984; Cubitt, 1987]. To determine if other stool-shed viruses are similarly affected by such storage, fecal specimens previously examined in this laboratory by EM and observed to contain virus particles were reexamined after 6–10 years of storage at −70° to −85°C.

MATERIALS AND METHODS

Fecal Specimens

The specimens that were reexamined were part of a collection of EM-positive (for virus particles) fecal specimens that had been acquired from waterborne gastroenteritis investigations [Hopkins et al., 1984; Williams, 1985], from a multiyear infection surveillance study [Camann et al., 1985], and through other virus-related research activities. Most of the specimens were collected within the United States, but several came from Egypt.

The specimens contained virus particles of five distinct morphological types, including astrovirus, calicivirus, small round structured virus (SRSV) [Caul and Appleton, 1982], adenovirus, and rotavirus. Morphologically featureless viruses, such as those of the enterovirus and parvovirus groups, were not included in this study. However, stored specimens containing two morphologically distinct virus-like particles, coronavirus-like particles (CVLPs) and T = 19 virus-like particles, were included in the reexamination.

The SRSV-containing stools included three specimens that had been identified as Norwalk-positive with a Norwalk virus radioimmunoassay (RIA) devel-

Accepted for publication July 28, 1989.

Address reprint requests to Fred P. Williams, Jr., Environmental Monitoring Systems Laboratory, USEPA, 26 West M.L. King Dr., Cincinnati, OH 45268.

TABLE I. EM Examination of Stool Specimens After Long-Term Storage at −70°C to −85°C.

Virus	Size (nm)	Morphological characteristics	No. of specimens examined	Years in storage (No. of specimens)	Number of specimens with characteristics still visible
Astrovirus	28–30	Starlike surface configurations	2	6 (1), 8 (1)	2
Calicivirus	30–40	Dark-staining cup-shaped depressions	1	6 (1)	1
Small round structured virus	27–40	Ragged-edged with poorly defined surface structure	4	7 (2), 8 (1) 9 (1)	4
Rotavirus	65–75	Wheel-like appearance when stain penetrated	4	8 (3), 10 (1)	4
Adenovirus	70–90	Large icosahedral shape, well defined capsomers	5	6 (1), 8 (4)	5
Coronavirus-like particles[a]	Variable	Highly pleomorphic distinctive fringe	5	6 (1), 7 (2), 8 (1), 9 (1)	5
T = 19 virus-like particles[a]	65–70	Capsomers in T = 19 arrangement, darker staining vertices	1	8 (1)	1

[a]It remains to be determined whether these virus-like particles are human viruses.

oped at the University of Massachusetts Medical School [Blacklow et al., 1979]. A fourth specimen containing SRSV particles was Norwalk-negative with the RIA but was not further characterized. All four specimens possessed the structured, ragged-edged particles described by Caul and Appleton [1982] at the time of the original examination.

All specimens had been stored at −70°C to −85°C for at least 6 years, and one for as many as 10 years, prior to reexamination. (The specimens were initially stored in a freezer at −70°C but were later transferred to a −85°C freezer when the original unit malfunctioned. At the time of the transfer, all specimens had been stored for at least 1 year at −70°C.) The specimens were stored as collected, without the addition of a storage medium, except for one SRSV-containing specimen that had been suspended in a transport medium (buffered glycerol saline) before shipment to this laboratory. Sterile distilled water had been added to two specimens to prevent drying. All specimens except for four (of five) containing CVLPs were from individuals afflicted with gastroenteritis. Those four CVLP-containing stools were nonillness specimens.

Electron Microscopy

Direct negative-stain examination of the fecal specimens was carried out as previously described [Williams, 1985]. Briefly, a turbid suspension of stool was made in distilled water and a drop applied to an EM grid with carbon support film. After 1 min, excess material was removed from the grid with filter paper. The grid was rinsed with one or two drops of distilled water and then stained with 2% phosphotungstic acid, pH 7. Excess stain was removed with the filter paper. After drying, the grid was examined at 80 kV with a JEOL JEM-100CX transmission electron microscope. The procedure was the same as that used in the original examination of the specimens.

RESULTS

The results of the reexamination of fecal specimens are shown in Table I. Virus particles were visualized in each of the specimens reexamined. In each specimen, the particles retained sufficient structural integrity to permit morphological identification. This identification was consistent with the original identification made 6–10 years earlier.

Astrovirus particles were identified by the starlike configurations exhibited on suitably oriented particles (Fig. 1A). Characteristic particles were apparent in each of the two specimens reexamined. However, recognition of the starlike configurations depended greatly on a favorable negative-stain environment.

In the single calicivirus-containing stool reexamined, the observed virus particles were striking in their definition (Fig. 1B). They exhibited well defined cup-like depressions and at times revealed a characteristic Star of David (see arrowhead, Fig. 1B), a configuration that is not exhibited by astroviruses.

Much less well defined than the calicivirus particles, but still morphologically distinct, were the SRSV particles observed in the reexamined stools (Fig. 1C). These particles possessed a visible surface structure. They were ragged-edged and often exhibited a lacy or lattice-like appearance. No morphological differences were noted between the SRSV particles present in the three Norwalk RIA-positive and single Norwalk RIA-negative specimens.

Rotavirus, a larger virus, was easily distinguished by the wheel-like appearance of stain-penetrated particles. Rotavirus particles were recognized in four reexamined specimens. The pictured particles (Fig. 1D) were from the stool specimen that had been stored the longest; it had been stored for 10 years.

Adenovirus, another large virus, was identified by its large icosahedral shape and well visualized capso-

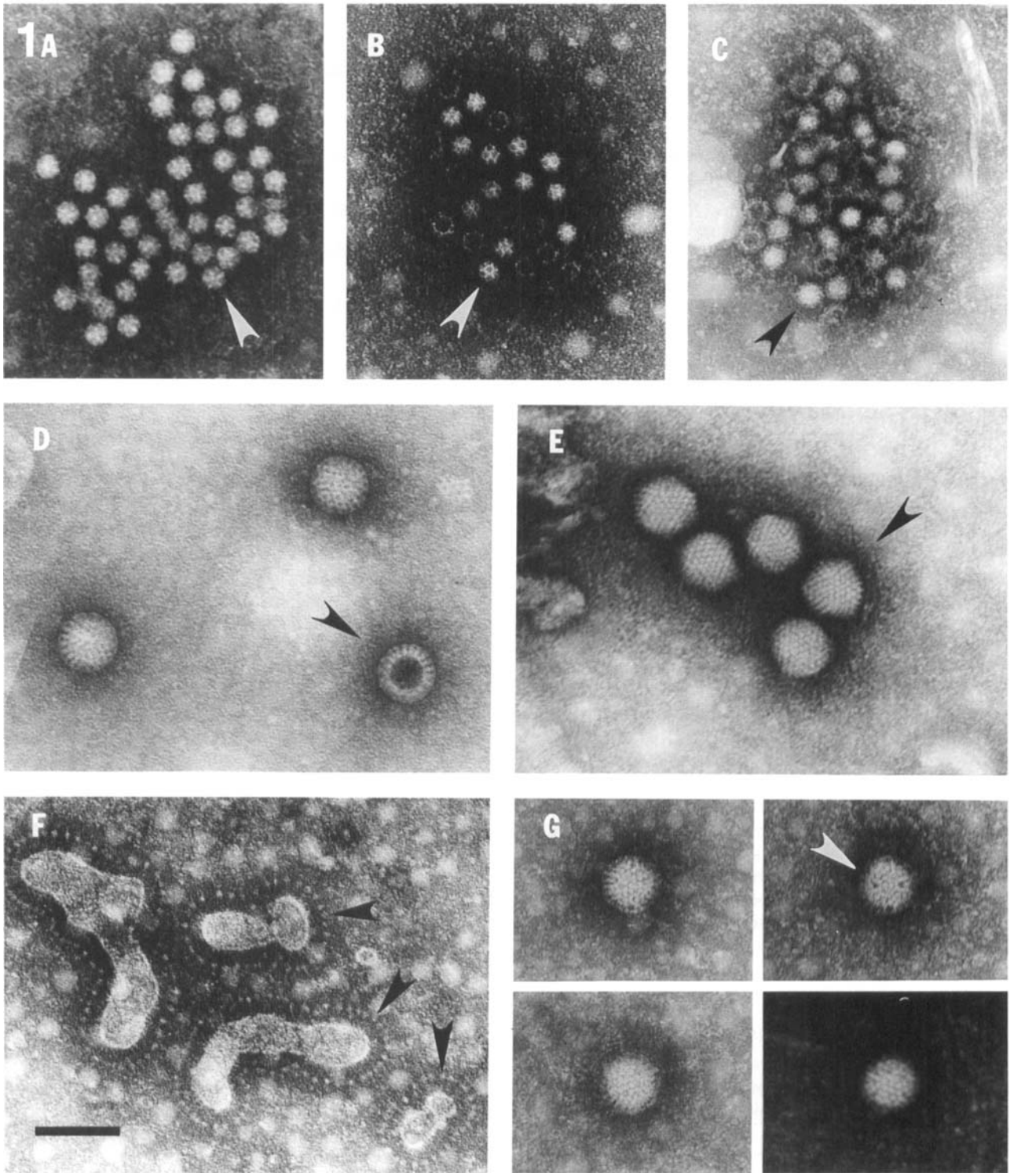

Fig. 1. Electron micrographs of the virus particles observed in stool specimens after long-term storage at −70°C to −85°C. **A:** Astrovirus after 6 years. **B:** Calicivirus, 6 years. **C:** SRSV (Norwalk RIA-positive), 7 years. **D:** Rotavirus, 10 years. **E:** Adenovirus, 6 years. **F:** CVLPs, 7 years. **G:** T = 19 virus-like particles, 8 years. Particularly characteristic particles are indicated with arrowheads. Bar = 100 nm for all micrographs.

meric construction (Fig. 1E). Characteristic adenovirus particles were recognized in five specimens.

The two virus-like particles were also identified in the reexamined stools. Characteristic CVLPs were highly pleomorphic and exhibited a fringe of projections surrounding the particles (Fig. 1F). CVLPs were recognized in five reexamined specimens. Although the true nature and significance of CVLPs have not been determined [Macnaughton and Davies, 1981; Schnagl et al., 1987], they are among the most common virus-like particles encountered in stools by EM.

T = 19 virus-like particles (Fig. 1G) were visualized after 8 years of storage. During a study of swimming-related illness in Egypt [Williams, 1985], these particles were observed in the stool of a young girl afflicted with gastroenteritis. Characteristic particles exhibited capsomeric construction with T = 19 icosahedral symmetry and displayed vertices (at the fivefold axes of rotational symmetry) that usually stained darker than other areas. As with the CVLPs, the significance of the T = 19 virus-like particles remains to be determined.

DISCUSSION

Of the five morphological types of stool-shed viruses and two types of virus-like particles reexamined in this study, all proved recognizable in specimens that had been stored a minimum of 6 years. The characteristic morphology of rotavirus was observed to be retained even after 10 years of storage at −70°C to −85°C. A similar observation has been reported by Albrey and Murphy [1976], who found that rotavirus particles present in pooled stool specimens retained their characteristic features after storage at −20°C for at least 9 years.

We did not expect to find remarkably distinct calicivirus particles present after 6 years of storage. Humphrey et al. [1984] have reported that the calicivirus particles they observed in stools lost their characteristic morphology after only a few weeks of storage at −70°C. Cubitt [1987] recommended holding stools to be examined for calicivirus at 4°C. The present study does not support these findings, although it must be noted that only one calicivirus-containing stool was reexamined in this study.

Although some degradation of virus particles may have occurred during storage, or through freezing and thawing, it was not significant enough to prevent the recognition of characteristic virus particles in any of the stools reexaminded in this study. The results of this study indicate that storage of stool specimens at ultralow temperatures presents no significant problems for the morphological identification of viruses.

REFERENCES

Albrey MB, Murphy AM (1976): Rotaviruses and acute gastroenteritis of infants and children. Medical Journal of Australia 1:82–85.

Blacklow NR, Cukor G, Bedigian MK, Echeverria P, Greenberg HB, Schreiber DS, Trier JS (1979): Immune response and prevalence of antibody to Norwalk enteritis virus as determined by radioimmunoassay. Journal of Clinical Microbiology 10:903–909.

Camann DE, Graham PJ, Guentzel MN, Harding HJ, Kimball KT, Moore BE, Northrop RL, Altman NL, Harrist RB, Holguin AH, Mason RL, Becker Popescu C, Sorber CA (1985): Health Effects Study for the Lubbock Land Treatment Project. Lubbock Infection Surveillance Study. Research Triangle Park, NC: United States Environmental Protection Agency, pp 1–638.

Caul EO, Appleton H (1982): The electron microscopical and physical characteristics of small round human fecal viruses: An interim scheme for classification. Journal of Medical Virology 9:257–265.

Cubitt WD (1987): The candidate caliciviruses. In Bock G, Whelan J (eds): "Novel Diarrhoea Viruses." Chichester: John Wiley & Sons, pp 126–138.

Hopkins RS, Gaspard GB, Williams FP Jr, Karlin RJ, Cukor G, Blacklow NR (1984): A community waterborne gastroenteritis outbreak: Evidence for rotavirus as the agent. American Journal of Public Health 74:263–265.

Humphrey TJ, Cruickshank JG, Cubitt WD (1984): An outbreak of calicivirus associated gastroenteritis in an elderly persons home. A possible zoonosis? Journal of Hygiene 93:293–299.

Lennette DA, Melnick JL, Jahrling PB (1980): Clinical virology: Introduction to methods. In Lennette EH, Balows A, Hausler WJ Jr, Truant JP (eds): "Manual of Clinical Microbiology, 3rd ed." Washington, DC: American Society for Microbiology, pp 760–771.

Macnaughton MR, Davies HA (1981): Human enteric coronaviruses. Archives of Virology 70:301–313.

Schnagl RD, Brookes S, Medvedec S, Morey F (1987): Characteristics of Australian human enteric coronavirus-like particles: Comparison with human respiratory coronavirus 229E and duodenal brush border vesicles. Archives of Virology 97:309–323.

Williams FP Jr (1985): Virus-like particles with T = 19 icosahedral symmetry in a human gastroenteritis stool. Micron and Microscopica Acta 16:173–178.