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An Electron Microscopical Investigation of Faecal Small Round Viruses

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A retrospective study of small round featureless viruses (SRVs) initially identified by negative-staining electron microscopy of stool samples was performed. A variety of techniques, including immunoelectron microscopy and caesium chloride gradient centrifugation, was applied in an attempt to classify further these viruses.

Over a four-year period, 64 SRV-positive samples were reported (1.8% of the stool samples sent for electron microscopy and 6.2% of the total number of positive samples), of which 53 were available for further study. A significant degree of misclassification was found. Viruses previously identified as SRVs were shown to be astrovirus (n = 14), calicivirus (n = 2), and "Norwalk-like" virus (n = 1). The majority of the 36 remaining samples were identified as parvovirus-like (n = 27) (75%), 14 of which were associated with the presence of adenovirus particles. Enteroviruses (n = 3) and hepatitis A virus (n = 1) were infrequently detected. The remaining viruses (n = 5) could not be adequately classified.

Parvovirus may be the predominant SRV associated with acute diarrhoeal disease in childhood.

Key words: small round virus, electron microscopy, gastrointestinal

INTRODUCTION

The use of negative-staining electron microscopy on stool specimens has been a major factor in detecting many of the putative causal agents of acute nonbacterial gastroenteritis [Madeley, 1986]. Many types of virus have been identified by this method, including rotavirus, adenovirus, astrovirus, calicivirus, and the "Norwalk-like" viruses, each having their own characteristic morphology [Madeley, 1986]. Indeed it is their morphology as seen by electron microscopy that allows their rapid identification.

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However, there remains a group within which the viruses appear morphologically similar in the electron microscope. They are seen as small (20–30 nm) featureless particles that are devoid of clear surface structure. These are classified as the small round viruses (SRVs), as they cannot be placed in any of the structured-virus groups [Caul and Appleton, 1982; Madeley, 1986]. Little is known about the relative prevalence of viruses within this classification or their clinical association [Dolin et al, 1987], although two families of SRVs are thought to occur in stools, the picornaviridae and the parvoviridae. Within the picornaviridae only enteroviruses have been found in stools [Madeley, 1986].

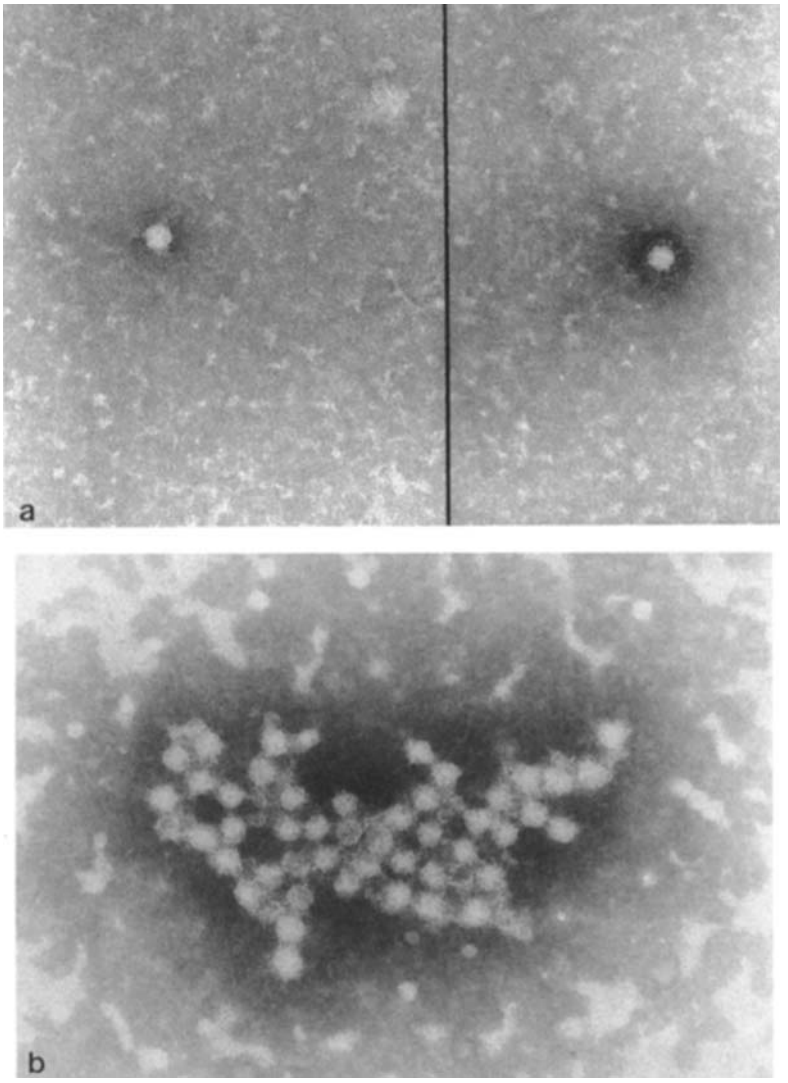

Fig. 1. Positive identification of astrovirus by LPIEM. **a:** Negative-staining EM preparation showing a featureless astrovirus. ×146,400. **b:** Agglutination of virus shown in A by antiastrovirus human convalescent serum. ×146,400.

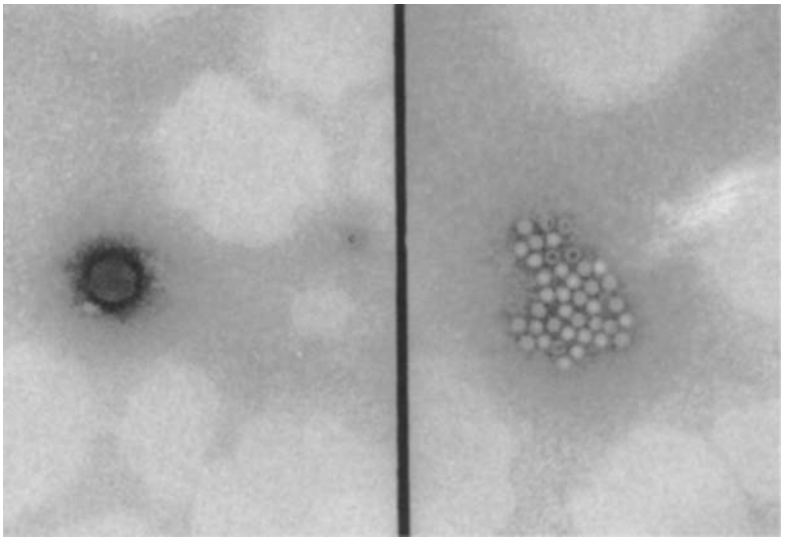

Fig. 2. Negative-staining EM preparation showing adenovirus with adeno-associated virus. ×122,000.

Defective parvoviruses, which require a co-infection with adenovirus for their complete replication, are known to occur in humans [Hoggan, 1970]. These are termed adeno-associated viruses (AAVs). Nondefective parvovirus-like particles have also been found in human faecal specimens [Caul and Appleton, 1982; Paver et al, 1974].

The aim of this study was to classify further the small round viruses detected in stool samples of children with acute diarrhoea using particle diameter measurement, immuno-electron microscopy [Anderson and Doane, 1973; Svensson and Bonsdorss, 1982; Gerna et al, 1984], and buoyant density determination [Paver et al, 1974; Siegl and Frosner, 1978; Feinstone et al, 1974].

MATERIALS AND METHODS

Stool preparations from children with acute diarrhoea are routinely screened for the presence of viruses by electron microscopy (EM) at the Queen Elizabeth Hospital for Children. The material used in the study consisted of all stools found to be positive for small round virus by routine direct negative-staining EM over the period January 1981 to February 1985. They had been suspended in distilled water, centrifuged at 1,500 rpm for five minutes to aid clarification, studied by EM, and stored at −20°C.

Halocarbon extraction was performed on freshly thawed stools. An equal volume of trichlorotrifluoroethane (BDH Chemicals Ltd, Poole, England) was added to the stool suspension. The tubes were agitated, clarified as above by a low-speed centrifugation, and allowed to settle for one hour. The supernatant aqueous phase was used for further examination.

Direct negative-staining EM was performed by placing a formvar/carbon-coated 400-mesh grid on a drop of supernatant, draining it on filter paper and staining with 3% phosphotungstic acid (PTA) (pH 6.6). The grid was drained, air-dried, and examined at a magnification of 34,000 in a Philips TEM 300. Particle diameter measurement was made on these preparations using an eyepiece graticule fitted in a binocular microscope (final

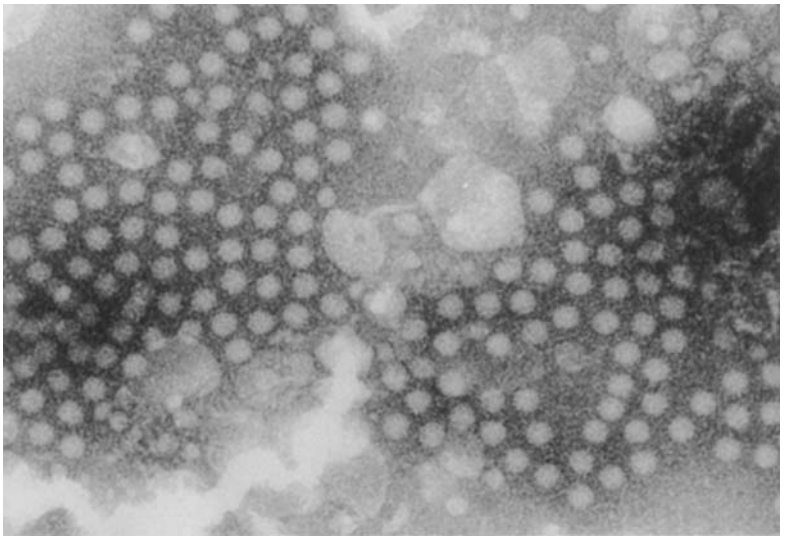

Fig. 3. Positive identification of hepatitis A virus (HAV) by LPIEM using anti-HAV IgM-positive serum. ×170,200.

magnification ×450,000). The EM was calibrated using a negatively stained bovine catalase crystal [Agar and Chescoe, 1974].

Immunoelectron Microscopy (Figs. 1–3)

Liquid-phase immunoelectron microscopy (LPIEM) was used to identify enteroviruses (including hepatitis A virus). Three antisera were used:

1. Convalescent human serum (1:10 dilution) positive for hepatitis-A-specific IgM by radioimmunoassay.

2. An enterovirus pool including antisera against all serotypes of poliovirus and coxsackie B virus, echoviruses (excluding types 4, 16, 21, 33, and 34), and coxsackie A virus types 7, 9, and 21. All antisera, apart from antiechovirus, which was used at 1:1 dilution, were mixed in equal volumes at 1:10 dilutions. Dilutions were made using phosphate-buffered saline (PBS). All were neutralising antisera obtained from the Department of Microbiological Reagents and Quality Control, Colindale.

3. Convalescent human antiastrovirus serum (1:100 dilution). This was obtained from a member of the nursing staff who acquired an astrovirus infection while on the infectious diseases ward.

Ten microliters of the stool suspension was mixed with 10 μl of the appropriate antiserum and incubated at 37°C for 30 minutes. Five microliters of this mixture was placed on a grid, drained, and negatively stained as before. A positive result was obtained if clumping of virus was seen on the test grids but not on control grids (PBS substituted for antiserum). Positive controls were included to ensure that the antisera were functional.

Solid-phase immunoelectron microscopy (SPIEM) was subsequently used to confirm the results of LPIEM for enteroviruses (excluding hepatitis A virus) and to confirm

and partially subtype any astrovirus-positive results. The enterovirus serum pool was that used in the LPIEM. Rabbit antitype I astrovirus serum (kindly supplied by Mr. T. Lee, Oxford Public Health Laboratory) was used at a dilution of 1:1,000 to assess the frequency of type I astrovirus infections.

Five microliters of protein A (Pharmacia Uppsala, Sweden), at a concentration of 50 μg/ml, was placed on a grid, incubated for 20 minutes at room temperature, and drained. Antiserum was applied and the grid was incubated at room temperature for one hour. The antibody-coated grid was washed twice with 0.1 M PBS, stool suspension was applied, incubated for 40 minutes, washed once with PBS, and stained with 3% PTA. The mean number of particles per grid square (a total of five squares were examined per grid) was determined on this and an appropriate direct EM control grid. A positive result was obtained if a greater than five-fold increase in particle numbers was observed.

Buoyant Density

Suspensions in which the only information found was particle size were run on caesium chloride gradients to determine the buoyant density of the particles. Continuous gradients were formed by layering 0.3 ml of the stool supernatant onto 4.5 ml of 45% aqueous caesium chloride (Sigma Chemical Co., Poole, England) and running at 100,000g for 18 hours (4°C) in an MSE 65 Superspeed ultracentrifuge using a swing-out rotor. Five microliters of 0.3-ml fractions whose density lay between 1.2 and 1.5 g/ml were placed on agar-coated slides and left for one hour with a grid on each drop. Once the caesium chloride had been absorbed, the grids were stained with PTA as above and allowed to air dry. For each grid, the mean number of particles per grid square (five squares per grid) was determined. This result was plotted against fraction density. The density of the fraction(s) containing the most virus was taken to represent the buoyant density of the virus.

Classification

Classification of the small round viruses found was made using the following criteria: (1) morphology and particle diameter, (2) presence/absence of adenovirus in the stool, (3) reaction with a specific antiserum or human convalescent serum, and (4) buoyant density if 1–3 did not permit positive identification.

The morphology, buoyant density (SRVs only), and mean particle diameters of the viruses were defined as follows. Parvovirus: hexagonal, no prominant surface features, 22 nm in diameter, 1.38–1.40 g/ml density [Paver et al, 1974]. These were termed parvovirus-like in the absence of buoyant density measurements. Adenoassociated virus: hexagonal, 23 nm in diameter, density 1.38–1.40 g/ml, present with adenovirus [Hoggan, 1970]. Enterovirus: no surface features, 27 nm in diameter, 1.32–1.37 g/ml density [Schild et al, 1987]. Astrovirus: "star-like" morphology, 28 nm in diameter [Madeley and Cosgrove, 1975]. Calicivirus: "pitted" morphology, 33 nm in diameter [Madeley, 1986]. "Norwalk-like" viruses: ill-defined surface morphology, 33 nm in diameter [Madeley, 1986].

No attempt was made to isolate virus in cell culture.

RESULTS

Over the four-year study period, 64 SRV-positive stools were identified on morphological grounds. These represented 1.8% of the 3,490 stool samples sent for EM and 6.3% of the positive samples. Five of these could not be traced, and in six, insufficient virus was

present to allow further classification. These 11 samples were therefore excluded from the study.

Fourteen of the remaining stools had both SRVs and adenovirus particles detected by direct EM. These 14 SRVs were classified as AAVs because of this association and because of their characteristically small size (21–25 nm).

The use of LPIEM for hepatitis A yielded one positive sample. The buoyant density of this virus was determined as 1.31 gcm^{-3}. Convalescent serum from this patient was shown to contain hepatitis-A-specific IgM. No enterovirus positives were identified when LPIEM and SPIEM were performed using the enterovirus serum pool, even though positive results were obtained when testing previously frozen known enteroviruses.

Examination of the controls for the immunoelectron microscopy tests allowed the positive identification of two caliciviruses and one "Norwalk-like" virus. It also prompted the use of LPIEM for astrovirus, as two stool samples contained virus particles that exhibited characteristic "star-like" morphology. Two "suspect" samples, as well as 12 others, were found to be astrovirus positive by LPIEM. Astrovirus SPIEM identified 6 of the 14 as type *1* astrovirus-positive samples.

Size measurements of all the positive virus identifications were concomitant with the other findings. Further size measurements were performed on the remaining 22 unclassified stools. The buoyant density of ten of these samples that contained sufficient virus was determined.

As a result of size measurements 13 parvovirus-like positive samples were identified. The mean diameter in 11 samples was 23 or 24 nm, the remaining two had diameters of 22 and 25 nm, respectively. The buoyant densities of four of these lay in the range 1.38–1.40 gcm^{-3}, establishing them as parvoviruses rather than parvovirus-like particles. Three enterovirus-positive stools were found, the particles having mean diameters of 27, 28, and 30 nm, respectively. One of these had a buoyant density of 1.33 gcm^{-3}.

A final group of five samples was found in which further identification was not possible. In four of the samples, viruses were present that had mean diameters between 22 and 24 nm, the remaining sample contained a virus 26 nm in diameter. The buoyant densities of the former four viruses varied between 1.31 and 1.33 gcm^{-3}. It was not possible to establish a precise buoyant density for the latter, as particles were present in fractions having densities ranging from 1.29 to 1.36 gcm^{-3}.

DISCUSSION

Faecal viruses are classified as small round viruses in the absence of clear morphological information to the contrary. It is, therefore, reasonable to assume that if the morphology of a small round structured virus is obscured, it could easily be classified as an SRV. Indeed, this does appear to have happened, especially in the case of astrovirus infections. Fourteen of the fifty-three samples initially classified as SRVs were identified as astrovirus-positive samples, indicating a potential degree of misclassification when using negative-staining EM alone. The use of convalescent human serum to identify astrovirus is not an ideal method, as it may contain antibodies to other viruses. However, it came from an adult, who had been well previously, following a documented astrovirus infection, and was diluted 1 in a 100. Additionally, two of the astrovirus-positive samples were identified on morphological grounds and half of the results were confirmed using type 1 astrovirus-specific rabbit antiserum. It can therefore be confidently stated that misclassification had occurred.

Caul and Appleton [1982] have observed that featureless astrovirus particles occur, and in the original description of the virus it was stated that 90% of the particles do not show the typical "star-like" appearance [Madeley and Cosgrove, 1975]. This may be a common but unappreciated problem in identification. Using SPIEM 43% of these astrovirus-positive particles were found to be type I. This is a lower incidence than might be expected, as Kurtz and Lee [1984] estimated that 77% of all astrovirus infections are type I. The lower incidence may simply be due to a small sample size.

Size and buoyant density allowed three enteroviruses to be identified, although none of them reacted with any of the antisera listed. This indicates that these viruses are neither coxsackie B or poliovirus and that they may be less common echoviruses or coxsackie A viruses. It is certainly unlikely that viral antigenicity has been lost, as positive control virus similarly stored at −20°C for several years was still reactive with the antisera used. It is possible that they are enteroviruses that are difficult to grow in cell culture, a feature common to the majority of gastrointestinal viruses detectable by electron microscopy. Antisera against them would not therefore be readily available.

Adeno-associated parvovirus-like particles were present in fourteen stool samples, and thirteen samples contained particles of a similar size when no adenovirus particles were identified. Buoyant density was measured in four of the thirteen latter samples to confirm their identity as parvoviruses. The other samples have been termed parvovirus-like in the absence of buoyant density results. It is possible that the latter viruses are true nondefective parvoviruses or that they are defective adeno-associated parvoviruses and the adenovirus particles are present in insufficient numbers for detection by electron microscopy. Tissue culture is required to investigate this point. Parvovirus-like particles therefore composed the largest group within the small round viruses.

In four cases viruses that were 22–24 nm in diameter, with buoyant densities lying within the range 1.31–1.33 gcm^{-3}, were found. These are therefore too small to fit in our classification of enteroviruses, yet their buoyant densities are too light for them to be classified as parvoviruses. It is possible that they are small enteroviruses or isometric, tailless bacteriophages, although they do not resemble the known phages MS-2 and oX174 [Paver et al, 1974]. The unclassified virus of 26-nm diameter is probably an enterovirus, but the uncertain buoyant density and the absence of any positive reaction with antisera preclude any definite identification.

Mean particle diameter measurement was of considerable use in the classification of SRVs. There are inherent inaccuracies with this method, as electron microscope magnification can fluctuate with time, even if hysteresis is minimised [Agar and Chescoe, 1974]. However, the use of an in-built graticule for direct measurement should reduce errors by removing the photographic process, and all measurements were made on a single electron microscope by one operator. The range of sizes given for SRVs [Caul and Appleton, 1982] are of little use in classification, particularly as it is not clear if the values given represent the actual range of sizes of all the SRV particles studied or the range of the mean results for the various viral types. When mean diameters have been quoted, picornavirses (27 nm) [Schild et al, 1987] are larger than parvoviruses (22 nm) [Paver et al, 1974] (20 nm) [Tinsley and Longworth, 1973], and this distinction was used as a means of classification. We found the majority of SRVs (excluding astrovirus) were 21–24 nm in size, although a few larger viruses were found, including one hepatitis A virus. Our experience indicates that astrovirus should be excluded before a virus of greater than 26 nm is classified as an SRV.

In conclusion, only thirty-six of fifty-three samples originally said to be positive for SRVs actually contained small round featureless viruses. Of these thirty six, 75% were parvovirus-like (including AAVs), 11% were enteroviruses (including one hepatitis A virus), and 14% remained unclassified. The application of cell culture techniques should facilitate further classification, and attention should be directed toward parvoviruses as potential causes of sporadic cases of acute gastroenteritis in children.

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