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An ion-exchange capture technique for routine identification of faecal viruses by electron microscopy

A.A. Codd and H.K. Narang

Public Health Laboratory, Institute of Pathology, Newcastle General Hospital, Westgate Road, Newcastle on Tyne NE4 6BE, U.K.

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Faecal specimens from 520 patients with non-bacterial, gastroenteritis were examined by electron microscopy using four methods. These were (1) a direct dip method, (2) low-speed centrifugation, (3) ultracentrifugation and (4) a calcium phosphate method. The calcium phosphate method combined with low-speed centrifugation (750 × g, 2,100 × g) was considered overall best. The calcium phosphate method makes it possible to handle a large number of faecal specimens by saving considerable time and labour.

Introduction

A major problem in the examination of faecal material for virus particles by electron microscopy is the presence of contaminating substances at concentrations that give rise to an unacceptable background. The purification and concentration procedures currently employed depend primarily on particle size and density (Stain et al., 1972; Middleton et al., 1974). In most methods centrifugation at low speed is followed by high-speed centrifugation, and may also involve a density gradient for concentration of particles (Flewett et al., 1973, 1974; Madeley et al., 1977; Mathan et al., 1975). There are a number of ion-exchange substances used to purify virus particles and their subunits in column. The basic property of the net surface charge of the virus particle has been largely overlooked as a method of purification of samples for electron microscopy. Calcium phosphate has been used to purify and concentrate viruses and their related soluble antigen by ion-exchange chromatography in column (Taverne and Wildy, 1959; Taverne et al., 1958). We have developed an ion-capture method which has proved versatile in the routine purification of virus particles from faecal samples for examination by electron microscopy.
Materials and Methods

Calcium phosphate
The calcium phosphate (CaHPO₄·2H₂O) was prepared after the methods of Tiselius et al. (1956). Equal volumes of 0.3 M CaCl₂·H₂O (11.1 g in 200 ml) and 0.3 M Na₂HPO₄ (14.2 g in 200 ml) were run at the same rate (about 120 drops/min) from two separate flasks into a flask containing 100 ml of water and a mechanical magnetic stirrer. The resulting coarse floccular precipitate of calcium phosphate was allowed to settle and washed four times by decantation with distilled water. The precipitate was then stored as a suspension in 0.15 M phosphate-buffered saline (PBS, pH 7.2) at 4°C and used as required.

Faecal specimens
We obtained faecal specimens for electron microscopic examination over a period of 1 yr from 520 patients with non-bacterial gastroenteritis. Most of the specimens were collected within 1-3 days of onset of the disease and all specimens were examined using four routine methods: (1) a direct dip method, (2) low-speed centrifugation, (3) ultracentrifugation as described in detail previously (Narang and Codd, 1981) and (4) by a calcium phosphate method.

Method 1 A 15-20% suspension of faeces was prepared in PBS by shaking for 2-3 min in a Griffin flask shaker at maximum speed. An aliquot of the mixture was allowed to stand for 20 min at 4°C to permit the gross debris to sediment. A drop of the mixture was placed on Fomavar-carbon-coated grid, and after 2-3 min the surplus fluid was withdrawn off with a filter paper and the grids negatively stained with 2% phosphotungstic acid (PTA, pH 6.6).

Method 2 The faecal homogenates from method 1 were diluted to a 2-5% suspension and centrifuged at 750 × g for 10 min in an MSE (Measuring and Scientific Equipment Ltd., Sussex, England) bench centrifuge to remove the debris. The supernatant fluid was collected for the preparation of grids as described previously (Narang and Codd, 1979). Briefly, Fomavar-carbon-coated grids were placed at the bottom of flat bottom plastic tubes containing 200 μl of distilled water. Five to seven μl of the supernatant fluid were expelled into each tube. These tubes were then spun horizontally for 30 min at a speed of 3700 rpm (2100 × g) in an MSE bench centrifuge. After the run the grids were removed, dried and negatively stained with PTA.

Method 3 Five ml of the faecal suspension were spun at 3000 rpm for 30 min in the MSE bench centrifuge and after the run the supernatant fluid was ultracentrifuged (30,000 × g) for 2 h and the deposits were used to prepare grids.

Method 4 About 1 ml of the faecal supernatant fluid spun at 750 rpm was taken in a 60×7 mm Dreyer's tube and four drops of calcium phosphate, well mixed before use, were added to each tube. The mixture was left in the tube for 10 min for
adsorption, with gentle mixing about every 3 min. After the end of the adsorption time the calcium phosphate was allowed to settle to the bottom of the tube (about 5 min) and the fluid decanted and discarded. The calcium phosphate was washed three times by filling the tube with PBS and then allowing the calcium phosphate to settle and the supernatant fluid decanted. Finally, 100 µl of saturated versene (ethylenediamine tetraacetic acid disodium salt, 9.8 g/100 ml, pH 7.2) were added to each tube to dissolve the calcium phosphate. The mixtures were used to prepare grids by a low-speed centrifugation technique (Narang and Codd, 1980). Five to seven µl of the dissolved calcium phosphate mixture were used to prepare the grids by low-speed centrifugation as described in method 2. The grids were stained with PTA and examined with an AEI electron microscope at a standard magnification of ×25,000.

Results

Of 520 specimens processed, 67 were found to be positive by more than one method (Table 1). 114 were positive by the low-speed centrifugation and calcium phosphate methods, 71 specimens contained rotavirus, 23 adenovirus, five coronavirus and 15 small round virus, of which five were found with rotavirus, and two with adenovirus. Many of the positive specimens were negative when grids were prepared by the direct drop method (1) as the distribution over the grids was uneven, and particles were not seen on all grid squares or all squares of the same grid. In the preparations by low-speed centrifugation (method 2) a number of particles were seen on most grid squares, except when excessive debris caused grids to be thick and black, and therefore required a second preparation. Grids prepared from dissolved calcium phosphate (method 4) were satisfactory, as only occasionally were some grids thick and black. There was much less cellular and bacterial debris using the calcium phosphate method. In all calcium phosphate preparations adenovirus, rotavirus and coronavirus particles were seen singly and in groups of two to three. A large proportion of specimens contained virus particles in clumps of variable sizes (Figs. 1–3). Some of these clumps contained over 1000 particles and in many of the clumps virus particles were heaped in the centre

TABLE 1

Number of positive specimens detected by the four different methods.

| Method             | 1  | 2  | 3  | 4  |
|--------------------|----|----|----|----|
| Adenovirus         | 16 | 23 | 21 | 23 |
| Rotavirus          | 51 | 71 | 71 | 71 |
| Small round virus  | 00 | 15 | 11 | 15 |
| Coronavirus        | 00 | 5  | 5  | 5  |
| Total              | 67 | 114| 108| 114|
Fig. 1. Negatively stained grid prepared from calcium phosphate adsorbed material by low-speed centrifugation. (a) Low magnification (grid bar = 500 nm) showing large rotavirus clumps (arrow heads) and single and small clumps (arrows). (b) High magnification of rotavirus clump showing part of central heap (grid bar = 100 nm).

Fig. 2a, b. Negatively stained grid prepared as in Fig. 1, showing single and clumped adenovirus particles in low (grid bar = 500 nm) and high magnification (grid bar = 100 nm). Note very little background cellular debris.
Fig. 3. Negatively stained grid prepared as in Fig. 1, showing a clump of small round virus particles (grid bar = 150 nm).

Fig. 4. Negatively stained grid showing coronavirus (grid bar = 100 nm). Note the rosettes effect.

(Fig. 3). The grids prepared by calcium phosphate adsorption appeared to contain more particles per grid square and the particles showed typical viral morphological features (Figs. 1–4). Measurement of the virus particles prepared by each method showed no size difference from the mean of the virus.

In the case of small round viruses, 20–30 nm diameter single particles were rarely observed. The clumped virus seen contained 5–15 particles, but often many of the clumps consisted of over 1000 particles (Fig. 3).

Discussion

Since there is no ‘catch-all culture system’ for faecal viruses, electron microscopy is widely used as a routine diagnostic method. The essential requirement for examination of faecal material for virus particles by EM is the removal of bacterial debris. Various preparative techniques have been used to achieve this, such as differential centrifugation (Stain et al., 1972; Middleton et al., 1974; Flewett et al., 1973, 1974; Madeley et al., 1977; Mathan et al., 1975) or the ammonium sulphate
method (Caul et al., 1978). All these methods require the use of ultracentrifugation, at 10,000 × g or more, which makes it difficult to examine large numbers of specimens in the routine laboratory. The calcium phosphate method is superior to the direct grid method and is on par with the ultracentrifugation method. The grids prepared from calcium phosphate adsorption/versene-solubilised material by the low-speed centrifugation method showed a number of significant advantages. Firstly, there are only two low-speed centrifugation steps (750 and 2100 × g) using a bench centrifuge, making it possible to handle a large number of specimens and thus saving considerable time and labour. The calcium phosphate captures all viruses previously seen by EM methods and is effective even when viruses are present as mixtures (five rotaviruses and two adenoviruses with small round viruses). It has not been possible to demonstrate enteroviruses (polio, Coxsackie and ECHO) as they require agglutination by monospecific antisera (Narang and Codd, 1980). Furthermore, the low-speed centrifugation preparation produced an even distribution of particles over the grid squares, making it easy to examine the grids and thus reducing the time spent on the EM examination. This is particularly helpful in the examination of the negative specimens. In addition, there was very little cellular or bacterial debris and the count of particles appeared higher, making morphological resolution clearer. The calcium phosphate method not only cleans the faecal suspension of cellular bacterial debris but at the same time concentrates the virus particles, hence less faecal material is required to do the test. The overall positive rate of 22% from unselected cases of gastrointestinal acute and non-acute illness represents a satisfactory detection rate, and is a good capture. The technique does not work well with frozen faecal material as the freeze–thaw cycle releases soluble protein which competes for the available ion sites on calcium phosphate.

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