AN IMPROVED METHOD FOR THE ROUTINE IDENTIFICATION OF FAECAL VIRUSES USING AMMONIUM SULPHATE PRECIPITATION

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Received 7 April 1978

1. Introduction

Various techniques have been used for the examination of faeces for the presence of virus particles. The early techniques involved the use of density gradients to clarify and concentrate small round virus particles prior to immune electron microscopy [1,2]. Since this early work the importance of rotavirus as a causal agent of gastroenteritis has been established by ultracentrifuging faecal extracts prior to examination [3-5]. Many other candidate viruses have been reported using a similar technique [6-12]. It is now well recognised that many of these virus particles occur in such large numbers that they can be detected by examination of grids prepared directly from crude stool preparations [13].

All these techniques, with the exception of the direct grid method which lacks sensitivity, are relatively time-consuming and may result in damage to virus morphology. Furthermore the deposition of particulate material may make virus recognition difficult. This is particularly important in the examination of faeces from gnotobiotic animals which have a high lipid content and require clarification through 40% sucrose [14].

Important advances have been made recently in the routine diagnosis of rotavirus infections using the fluorescent antibody technique for the examination of infected cell culture [15,16]. A similar fluorescent antibody system can be applied to astrovirus detection [17]. However there still remain other faecal viruses which cannot be routinely detected in this way. Thus until reliable cell culture systems are available for all known enteric viruses, electron microscopy remains the only "catch-all" system which will give maximum information on the presence and importance of viruses in enteric disease. The increasing demand for the routine examination of faecal specimens by electron microscopy led us to evaluate ammonium sulphate precipitation as a useful rapid method for their examination.

2. Materials and methods

2.1. Source of virus and test faecal samples

Faecal emulsions were prepared as a 10% suspension in Earle's saline containing 0.5% lactalbumin hydrolysate and 0.132% bicarbonate with phenol red. These were prepared from human faeces known to contain adenoviruses, astroviruses, parvoviruses, coronavirus types and rotaviruses. In addition 100 human faecal specimens, known to be positive or negative for viruses, were treated in the same way and examined under code. Preparations were also made from animal faeces known to contain porcine and bovine coronaviruses, bovine astroviruses and the bovine "Newbury agent" [14]. All suspensions were clarified at 3000 rev./min for 30 min and the supernatant removed and treated by the following methods.

2.2. Methods used prior to staining

Method A: Direct grids were prepared from clarified faecal emulsions by placing a grid on a drop of emulsion for 3 min and then negatively staining.

Method B: 4 ml of the clarified faecal emulsion
were ultracentrifuged at 50 000 g for 2 h in a Sorvall RC2B centrifuge using an SM24 rotor. The supernatant was removed and the deposit resuspended in 4 drops of distilled water.

Method C: 4 ml of the clarified supernatant were mixed with 30%, 40%, 50%, 60% and 70% saturated ammonium sulphate and left at 4°C for 1 h. The preparations were centrifuged at 10 000 g for 10 min, the supernatant carefully removed and the precipitate dissolved in four drops of distilled water. For some experiments the supernatant was ultracentrifuged at 50 000 g for 2 h and the deposit resuspended in four drops of distilled water.

2.3. **Negative staining technique**

Formvar coated grids (250 mesh) were placed on a drop of virus suspension and left for a minimum of 3 min. Grids were removed and excess fluid withdrawn with filter paper. The grids were then placed on a drop of distilled water for a further 3 min followed by negative staining with 1.5% phosphotungstic acid (pH 6.5). Grids were examined in an AEI 801 electron microscope at an indicated magnification of 63 000. A minimum of four grid squares were examined for each specimen.

3. Results

Preliminary studies on human adenoviruses, rotavirus and coronaviruses were undertaken to ascertain the concentration of ammonium sulphate necessary for maximum precipitation. Following precipitation with concentrations varying from 30%—70% saturation, the resulting deposits and ultracentrifuged supernatants were examined in parallel. Maximum precipitation was obtained with the 60% saturation. Further it was found that precipitation for 1 h was as effective as precipitation for 4 h. This assessment was based on the examination of the supernatants for residual virus. Precipitation was equally effective at 4°C or room temperature.

In order to determine the sensitivity of this precipitation procedure (Method C), comparisons were made with the direct grid procedure (Method A) and the routine ultracentrifugation procedure (Method B). The results showed that Methods B and C were of equal sensitivity and both were superior to the direct grid method (Table 1).

To test the efficiency of the new method in the diagnostic situation, the one hundred specimens were processed in parallel by Methods B and C. The resulting grids were examined under code and the results are shown in Table 2. It was noted that grids prepared from ammonium sulphate precipitates had less contaminating material.

Some animal faeces, known to contain viruses, were also examined. The “Newbury agent” (Fig. 1a), which was readily visualized in large numbers following ammonium sulphate precipitation, was not seen in grids made from the ultracentrifuged deposit. These latter grids were heavily contaminated with extraneous material making resolution difficult. It was found that the porcine and bovine enteric coronaviruses (Figs. 1b, 1c) were more easily recognised following the precipitation method. Furthermore the faecal sample containing the bovine enteric coronavirus also contained an astrovirus-like particle (Fig. 1d). The five or six pointed star structure seen in astroviruses was clearer in the precipitated material. Precipitation did not affect the morphology of the rotaviruses or adenoviruses (Fig. 1e, 1f). However the human enteric coronavirus (Fig. 1g) showed less damage by the precipitation method resulting in fewer collapsed particles with more projections on their surface. Fig. 1h shows the appearance of approx. 22 nm parvovirus-like particles which were also unaffected by the precipitation method.

| Virus          | Direct grid (Method A) | Routine ultracentrifugation (Method B) | Ammonium sulphate precipitation (Method C) |
|----------------|------------------------|----------------------------------------|-------------------------------------------|
| Rotavirus      | 10                     | 400                                    | 400                                       |
| Adenovirus     | 10                     | 100                                    | 100                                       |
| Coronavirus    | 10                     | 100                                    | 100                                       |

*Expressed as reciprocal of the last dilution of faecal emulsion which was found to be positive for virus by electron microscopy.*
TABLE 2

Results of the examination of 100 faecal specimens processed by the ultracentrifugation (Method B) and ammonium sulphate precipitation (Method C)

| Virus     | Number of positive identifications |
|-----------|-----------------------------------|
|           | By BOTH methods | by Method B | by Method C | Total |
|           | only            | only        | only        |       |
| Coronavirus | 18              | 0           | 0           | 18    |
| Rotavirus  | 27              | 0           | 0           | 27    |
| Adenovirus | 11              | 0           | 0           | 11    |
| SRV a      | 4               | 0           | 3           | 7     |

Total negative = 37

* SRV, small round virus-like particles, 22–25 nm in diameter and lacking any distinctive morphology.

4. Discussion

Electron microscopy of faeces is widely used as a diagnostic method and the preparative techniques prior to examination vary in their complexity. This investigation clearly showed that of the three standard methods evaluated, ammonium sulphate was superior to the direct grid method and of equal sensitivity to the ultracentrifugation method. However the ammonium sulphate method had a number of significant advantages. Firstly, as there was no high speed centrifugation involved, this reduced the background contamination of grids enabling easier recognition of virus particles particularly the small round viruses. Furthermore this is particularly relevant when faeces from gnotobiotic animals are to be examined since this material has a high lipid content. Secondly, morphological appearance was improved with some viruses, notably the human enteric coronaviruses. This is

![Fig. 1](image-url)
not surprising in view of the known effect of high speed centrifugation on coronavirus morphology [18, 19]. Thirdly, and most applicable to a diagnostic laboratory handling large numbers of specimens, is the considerable time-saving factor in their processing and the concomitant reduction in centrifuge wear.

The method described here, was evaluated in terms of a “catch-all” system for the detection of enteric virus particles. Clearly, where epidemiological data are required on any virus which has been shown to replicate in cell culture, the fluorescent antibody techniques are more applicable. However the majority of human enteric viruses cannot at present be cultivated. Electron microscopy thus remains the method of choice in the investigation and determination of the role of virus particles in enteric disease.

Acknowledgements

We wish to thank Dr. J. Bridger for providing the veterinary faecal specimens.

References

[1] Kapikian, A.Z., Gerin, J.L., Wyatt, R.G., Thornhill, T.S. and Chanock, R.M. (1973) Proc. Soc. Exp. Biol. Med. 142, 874–877.

[2] Paver, W.K., Caul, E.O. and Clarke, S.K.R. (1974) J. Gen. Virol. 22 447–450.

[3] Flewett, T.H., Bryden, A.S. and Davies, H. (1973) Lancet ii, 1497.

[4] Bishop, R.F., Davidson, G.P., Holmes, I.H. and Ruck, B.J. (1974) Lancet i, 49.

[5] Middleton, P.J., Szymanski, M.T., Abbott, G.D., Bortolussi, R. and Hamilton, J.R. (1974) Lancet i, 1242.

[6] White, G.B.B., Ashton, C.I., Roberts, C. and Parry, H.E. (1974) Lancet ii, 726.

[7] Mathan, M., Mathan, V.I., Swaminathan, S.P., Yesudos, S. and Baker, S.J. (1975) Lancet i, 1068–1069.

[8] Caul, E.O., Paver, W.K. and Clarke, S.K.R. (1975) Lancet i, 1192.

[9] Madeley, C.R. and Cosgrove, B.P. (1975) Lancet ii, 124.

[10] Appleton, H. and Higgins, P.G. (1975) Lancet ii, 1297.

[11] Madeley, C.R. and Cosgrove, B.P. (1976) Lancet i, 199–200.

[12] Appleton, H., Buckley, M., Thom, B.T., Cotton, J.L. and Henderson, S. (1977) Lancet ii, 409–411.

[13] Middleton, P.J., Szymanski, M.T. and Petric, M. (1977) Am. J. Dis. Child. 131, 733–737.

[14] Woode, G.N. and Bridger, J.C. (1978) J. Med. Microbiol. (in press).

[15] Banatvala, J.E., Totterdell, B., Chrystie, I.L. and Woode, G.N. (1975) Lancet ii, 821.

[16] Bryden, A.S., Davies, H.A., Thoulless, M.E. and Flewett, T.H. (1977) J. Med. Microbiol. 10, 121–125.

[17] Lee, T.W. and Kurtz, J.B. (1977) Lancet ii, 406.

[18] Caul, E.O., Ashley, C.R. and Egglestone, S.I. (1977) Med. Lab. Sci. 34, 259–263.

[19] Tajima, M. (1970) Arch. Ges. Virusforsch. 29, 105–108.