VIRAL AGENTS ASSOCIATED WITH NEONATAL DIARRHOEA AND THEIR DETECTION BY ELECTRON MICROSCOPY

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ABSTRACT

Simple techniques for diagnosis of enteric viral infections by direct electron microscopy of faeces are described. For best results, some degree of purification and/or concentration of faecal material is necessary. This can be adequately achieved by differential centrifugation. A number of viruses have been observed in the faeces of the domestic animal species and man. These include rotaviruses, coronaviruses, astroviruses, caliciviruses, adenoviruses, parvoviruses and enterovirus-like particles. Mixed infections with combinations of these viruses are extremely common. Caution should be exercised in interpretation of results. Detection of one of the above viruses in the faeces of an animal with diarrhoea does not necessarily indicate etiological significance.

INTRODUCTION

Within the last decade a number of previously unknown enteric viruses have been discovered. Most of these viruses cannot be isolated in cell cultures using conventional techniques, but occur in sufficient numbers in infected faeces to allow them to be detected directly by electron microscopy (EM). In this paper, methods for the direct examination of faecal material by EM are described and results obtained with this technique are reviewed.

MATERIALS AND METHODS

Direct EM examination of faeces

Method A. Make an approximately 15% suspension of crude faeces in 1% ammonium acetate or distilled water. Apply to a grid for a few minutes and stain.

Method B. Dilute faecal material approximately 1:5 to a final volume of about 15 ml in phosphate-buffered saline (PBS). Centrifuge at 3 000 g for 30 min to remove bacteria and gross debris. Collect the resulting supernatant and centrifuge at 91 000 g for 1 h. Resuspend the pellet in a few drops of 1% ammonium acetate or distilled water and examine.

Method C. To about 15 ml of a 15-20% suspension of faeces add an equal

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volume of the fluorocarbon, Arcton 113 (ICI Ltd, Runcorn, Cheshire, England). Mix thoroughly to form an emulsion. Separate the aqueous and Arcton layers by centrifugation at 3 000 g for 15 min. Remove the upper aqueous layer, ultracentrifuge as described in Method B and examine the pellet.

Immune electron microscopy of faeces

Prepare a clarified faecal suspension as described in Methods B or C above. Add an equal volume of serum diluted with PBS. Either a specific antiserum or pooled sera obtained from adult animals of the same species as the specimens under test may be used. If the titre of the serum is unknown, test it at several dilutions e.g. 1:50, 1:250 and 1:1 000, and subsequently use the dilution which gives the best clumping. Incubate the serum-virus mixture for 2 h at 37°C or overnight at 4°C. Centrifuge at 10 000 g for 30 min and examine the pellet.

Grids and staining

Carbon-coated 400 mesh grids are used routinely at Stormont. Specimens are stained for about 1 min with methylamine tungstate (EMscope Laboratories Ltd, Ashford, Kent, England) or 4% sodium phosphotungstate, pH 6.5.

General considerations

Usually it is necessary to prepare only one grid from each specimen unless they are obviously unsatisfactory. Normally at least 5 or 6 good squares of a 400 mesh grid should be examined before a negative result is reported. At a working magnification of about 40 000 x, this will take about 10 min.

It is advisable to photograph virus-like particles of uncertain identity, as some viruses are difficult to identify unequivocally on the screen. This is particularly important for inexperienced operators, as it allows their results to be checked.

RESULTS

In our experience, it is easier to find virus particles in the contents of the large intestine, caecum and rectum than in small intestinal contents. Consequently the former are the specimens of choice from a dead animal. Sometimes, particularly in the case of young pigs or broiler chickens, it may be difficult to identify animals with diarrhoea or to obtain a sample of faeces from them. In this case it is best to scrape obviously diarrhoeic material off the floor. Animals should be sampled as

soon as possible after the onset of diarrhoea as virus titres tend to be highest at this time. Material should be sent to the laboratory in clean, screw-topped, secure containers. No diluent is necessary for EM examination. Many veterinary practitioners submit material collected on cotton wool swabs. These are unsatisfactory for several reasons. They often contain insufficient material and usually yield inferior preparations. Also if submitted dry, virus isolation attempts are likely to be unsuccessful. It is advisable to ask practitioners to submit samples large enough to permit further investigation if new or unusual viruses are found e.g. 10 ml from a calf, 5 ml from a pig.

It should be remembered that EM is not a sensitive technique. Virus at a concentration of 10^5 particles/ml is just at the borderline of detection (Flewett, 1978). Fortunately, however, most of the enteric viruses are excreted in vast numbers in the faeces, 10^{10} rotavirus particles/ml is not uncommon. Method A is very simple and was first described by Middleton et al. (1977), who used it to detect rotaviruses, adenoviruses and a variety of small round viruses in human faeces. We have found it useful for screening bovine faeces for rotavirus and coronavirus. It is not as satisfactory for pig and avian faeces. Pig faeces appear to contain a lot more non-viral contaminating material than bovine faeces and enteric viruses tend to be present in lower numbers in avian faeces than mammalian faeces (McNulty et al., 1979a). For faeces from these species, some degree of purification and/or concentration is necessary. Method B is normally suitable for this purpose. However, if faeces contain a lot of lipid and difficulty is encountered in clarifying suspensions, fluorocarbon extraction as described in Method C may be used.

Small round viruses (calicis, astros etc) are best demonstrated following pelleting, which facilitates observation of groups of particles. Clumping may also be achieved by immune EM. However, it is necessary to use the antiserum at the correct dilution. If antibody is present in too high a concentration, the surface structure of the virus particles will be obscured. Either specific antisera or pooled adult sera may be used for immune EM. Pooled adult sera possess antibodies against a wide range of micro-organisms, so that as general diagnostic reagents they are more useful than hyperimmune sera. Furthermore they have the added advantage of ready availability.

Table 1 lists the viruses which have been detected in the faeces of

the domestic animal species and man by direct EM. It is hardly surprising that the greatest variety of viruses has been recognised in calves and humans, as these have been the most intensively studied species.

TABLE 1 - VIRUSES DETECTABLE IN FAECES BY DIRECT ELECTRON MICROSCOPY

	Calf	Pig	Lamb	Foal	Dog	Cat	Turkey	Fowl	Human
Rotavirus	+[1]	+[2]	+[3]	+[4]	+[5]	+[6]	+[7]	+[7]	+[8]
Coronavirus	+[9]	+[10,11]	+[12]	+[13]	+[14,30]	+[15]	+[16]		+[17,18]
Astrovirus	+[19]		+[20]				+[21]		+[22]
Calicivirus	+[19]								+[23]
Parvovirus					+[24]				?[25]
Adenovirus									+[26]
Enterovirus-like	+[27]	+[28]			+[5]		+[7]	+[7]	+[29]

1 Mebus et al. (1969)
2 Rodger et al. (1975)
3 Snodgrass et al. (1976)
4 Flewett et al. (1975)
5 Eugster and Sidwa (1979)
6 Snodgrass et al. (1979)
7 McNulty et al. (1979a)
8 Flewett et al. (1973)
9 Stair et al. (1972)
10 Saif et al. (1977)
11 Pensaert and de Bouck (1978)
12 Tzipori et al. (1978)
13 Bass and Sharpee (1975)
14 Appel et al. (1978)
15 Hoshino and Scott (1980)
16 Ritchie et al. (1973)
17 Mathan et al. (1975)
18 Caul et al. (1975)
19 Woode and Bridger (1978)
20 Snodgrass and Gray (1977)
21 McNulty et al. (1980)
22 Madeley and Cosgrove (1975)
23 Madeley and Cosgrove (1976)
24 Eugster and Nairn (1977)
25 Kapikian et al. (1972)
26 Flewett et al. (1975)
27 McNulty et al. (1976)
28 McNulty, unpublished
29 Middleton et al. (1977)
30 Schnagl and Holmes (1978)

Rotaviruses. Next to bacteriophages, these are the most commonly encountered viruses in faeces. Regardless of the species of origin, all rotaviruses are morphologically indistinguishable (Woode et al., 1976; McNulty et al., 1979b). Intact particles are about 70 nm in diameter. They have been described as reovirus-like, but can be distinguished from reoviruses by virtue of their more clearly defined outer edge (Fig. 1.).

Furthermore, reoviruses are very rarely detected in faeces by direct EM, and are never present in the same vast numbers as rotaviruses. The outer shell of rotaviruses may be lost to produce particles which resemble orbiviruses and which are about 10 nm smaller than intact virions.

Coronaviruses. In recent years, there have been many reports of the detection of coronavirus-like particles in the faeces of animals and man, both with and without diarrhoea (Table 1). Some of these particles are morphologically different from classical coronaviruses, as exemplified by avian infectious bronchitis virus, most notably with respect to the surface projections. Caul and Egglestone (1979) have therefore suggested that there may be two subgroups of coronaviruses, one group with the classical petal-shaped projections and the other possessing projections consisting of thin stalks which terminate in spherical or teardrop-like knobs (Figs. 2, 3). However it is by no means certain that all of these coronavirus-like particles are indeed viruses, let alone pathogens.

Of the agents unequivocally shown to be coronaviruses, direct EM of faeces is a useful diagnostic procedure for bovine and canine enteric coronaviruses. The best characterised pig enteric coronavirus is transmissable gastroenteritis (TGE) virus. However, although TGE virus has been demonstrated in the faeces of experimentally infected pigs by immune EM (Saif et al., 1977), virus isolation and/or immunofluorescence are more commonly used for diagnosis (Pensaert and Debouck, 1978; Bohl, 1979). Recently, Pensaert and Debouck (1978) have isolated a new coronavirus from pigs in Belgium with an epizootic diarrhoea similar to TGE. This virus has not yet been isolated in cell cultures but can be detected in faeces by direct EM. Chasey and Cartwright (1978) described a coronavirus-like agent in the faeces of pigs in Britain with epidemic diarrhoea. However, demonstration of this agent by direct EM of faeces is unreliable. Neither TGE nor epidemic diarrhoea are present in Northern Ireland.

Difficulty in recognising coronaviruses will be encountered when partial or complete loss of the main morphological feature i.e. the corona of surface projections, has occurred. It should be remembered that the corona of some coronaviruses is very fragile and may be destroyed by freezing and thawing and/or prolonged storage.

Astroviruses. These are spherical viruses about 28 nm in diameter. Their main distinguishing feature is a star-shaped surface configuration. This can be clearly seen on only about 10% of the particles and may be either a

5- or 6-pointed star which occupies most of the surface of the particle (Fig. 4). The surface detail may be difficult to recognise on the screen, underlining the necessity to photograph particles of uncertain identity. Astroviruses have been detected in only a few animal species (Table 1).

Caliciviruses. Caliciviruses have a well developed star- or lattice-shaped surface configuration. However, unlike astroviruses, only 6-pointed stars are present. Furthermore, the calicivirus star has a central hollow whereas the centre of the astrovirus star is never filled with stain. Caliciviruses are slightly bigger (31 nm) than astroviruses and have a less well defined outer edge, with a scalloped as opposed to a circular outline (Fig. 5). The distinguishing features of caliciviruses and astroviruses have recently been described in detail by Madeley (1979). Enteric caliciviruses were first detected in the faeces of children with diarrhoea by Madeley and Cosgrove (1976). Calicivirus-like agents have also been isolated from calves (Woode and Bridger, 1978). These have a similar degree of pathogenicity for gnotobiotic calves as rotaviruses (J.C. Bridger, personal communication).

Parvoviruses. At present, direct EM is useful for diagnosing parvoviral enteritis only in dogs. This condition has assumed major importance all over the world during the last 2 or 3 years. Often the virus is present in sufficient numbers in the faeces of affected dogs to be detected easily by Method A (Fig. 6). Parvovirus infections can cause enteritis and diarrhoea in calves, but virus isolation is the recommended diagnostic method in this case (Storz and Leary, 1979). A number of human 'parvovirus-like' agents similar to and including the Norwalk agent have been described (Holmes, 1979). These do not occur in large quantities in the faeces and immune EM is usually necessary to allow them to be detected (Holmes, 1979). It is possible that these agents are not, in fact, parvoviruses. They are slightly larger (26-30 nm) than parvoviruses (18-26 nm) and have not yet been characterised biochemically.

Adenoviruses. Adenoviruses are sometimes found in very large numbers in the faeces of humans with diarrhoea. Normally these viruses cannot be isolated and grown in cell cultures (Flewett et al., 1975; Middleton et al., 1977). As far as we are aware no truly analagous situation has been described in animals. Adenoviruses are occasionally encountered in very small numbers in avian faeces, but these are probably the same viruses which can be readily isolated in cell cultures (McNulty et al., 1979a).

Enterovirus-like particles. Small round virus-like particles about 28 nm in diameter and without any obvious surface structure have been reported in the faeces of several animal species (Fig. 7, Table 1). These have not been isolated in cell cultures and their significance is unknown.

Other virus-like particles in faeces. Middleton et al. (1977) have described 30 nm, double-shelled, spherical virus-like particles in the faeces of children with gastroenteritis and diarrhoea. These particles have been called 'mini-reovirus', but at this stage their true nature is unknown. One suggestion is that they may be caliciviruses (Holmes, 1979). The fringed particles about 100 nm in diameter which Mebus et al. (1978) described in association with villous epithelial cell syncitia and diarrhoea in calves are not sufficiently distinctive morphologically to permit reliable diagnosis by direct EM. Similar particles are regularly encountered in the faeces of all the domestic species, both from normal animals and those with diarrhoea. For the same reasons, BVD/mucosal disease cannot be diagnosed with certainty by direct EM.

It is important to appreciate that mixed infections with combinations of the above viruses are extremely common e.g. rotavirus and coronavirus in calves, rotavirus and enterovirus-like particles in pigs and parvovirus and coronavirus in dogs. It is therefore essential to examine each grid thoroughly and not to stop as soon as the first virus is recognised.

DISCUSSION

It is obvious that direct EM examination of faeces is a potent tool in diagnosis of enteric viral infections. As most of the enteric viruses cannot be routinely isolated in cell cultures, it is the only catch-all system presently available for diagnosis. It permits very rapid diagnosis for important specimens and has the important advantage that mixed infections can be easily recognised. On the debit side, the basic equipment is expensive both to buy and to maintain. The number of specimens which can be processed by EM is very much lower than that possible using techniques such as ELISA. Another limitation is the insensitivity of the technique - about 10^6 virus particles/ml are required for consistent detection. Furthermore, as discussed above, some viruses may be difficult to recognise. However, it is likely that most of the blank spaces in Table 1 will be filled in during the next few years, and direct EM is probably the best way of searching for these viruses.

At present, interpretation of the results obtained by direct EM of

faeces is not easy. The pathogenicity of most of the viruses described here has not yet been adequately defined in the different animal species. Furthermore, there is the probability that different strains of the same virus may vary considerably in virulence. Although mixed infections occur very frequently in the field, there has been very little experimental work done using different combinations of viruses and mixed infections of viruses and enteropathogenic bacteria. Detection of a virus in the faeces of an animal with diarrhoea is therefore not sufficient grounds for ascribing etiological significance to it. Results should be considered in the light of bacteriological, and where possible, pathological findings.

When new virus-like particles are detected in faeces, the nature and significance of such particles can only be assessed by answering the following questions. How frequently are the particles detected in faeces? Are they observed in material from normal animals as well as those with diarrhoea? Does the period of their excretion coincide with that of clinical symptoms? Is it possible to demonstrate an antibody response in animals which have excreted the particles? Can they be isolated in cell cultures, intestinal organ cultures etc? Are they capable of infecting experimental animals, and if so, do such infections cause clinical disease?

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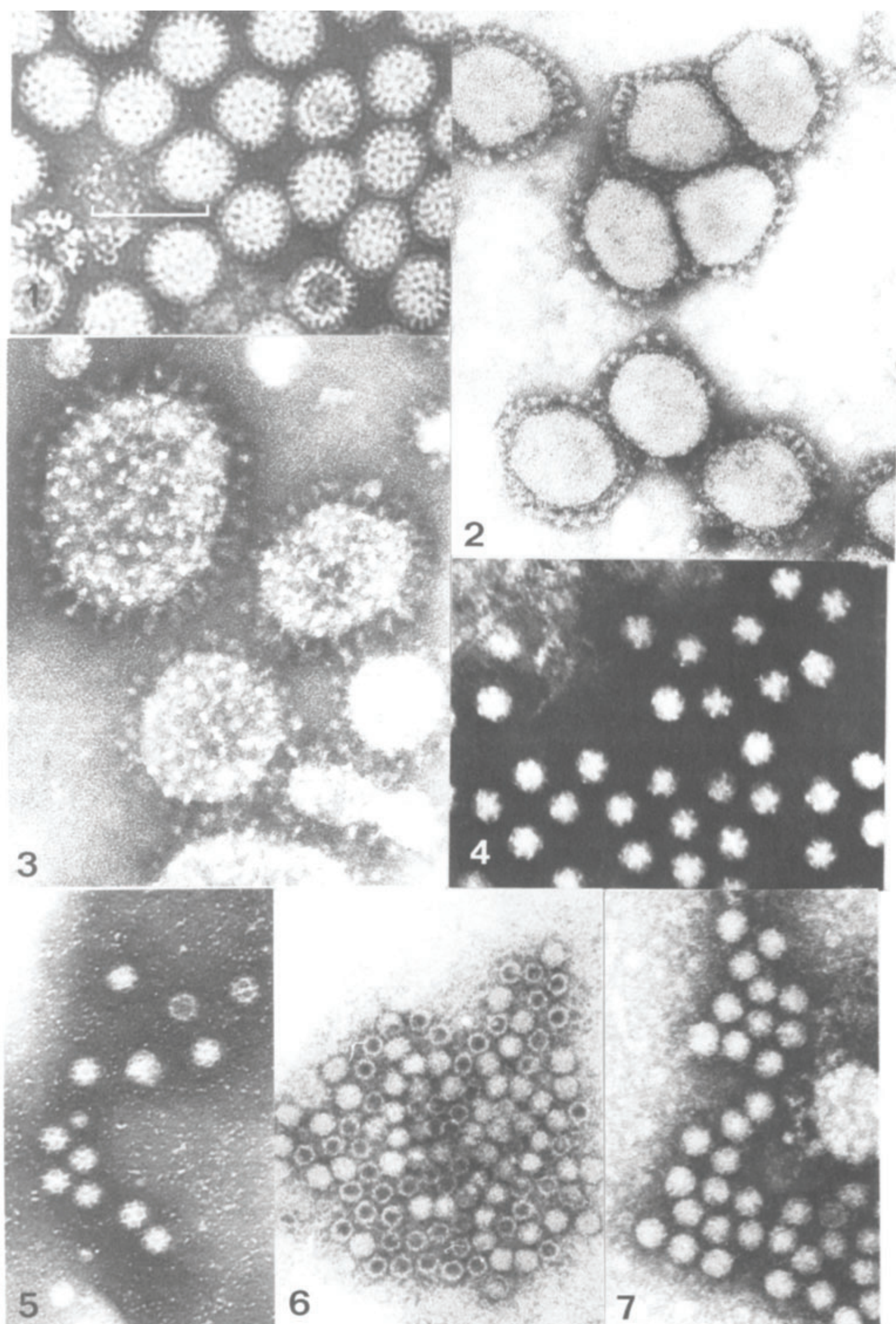
Figs. 1-7. Viruses and virus-like particles detected in faeces by direct electron microscopy. Bar represents 100 nm. Methylamine tungstate stain. 1. Bovine rotavirus. 2. Bovine enteric coronavirus. 3. Coronavirus-like particles from simian faeces. 4. Turkey astrovirus. 5. Human calicivirus. 6. Canine parvovirus. 7. Porcine enterovirus-like particles.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