Viruses in the stools

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SUMMARY It has long been possible to isolate viruses from the stools by culture, though the viruses found are rarely implicated in disease of the gut. In contrast, only recently has it been possible to identify viruses in the stools of patients with diarrhoea. Initially, such identifications were made by electron microscopy but the unsuitability of the microscope for large-scale screening has led to the development of other methods. The new methods have concentrated on rotaviruses but other viruses are also implicated and an overall view of the significance of finding a virus in any stool specimen has to take into account the evidence about all viruses, old and new.

Viruses are obligate parasites, which require living cells in which to replicate. They have been found in most organs of the body, but each virus normally shows considerable tissue tropism. Symptom-free carriage of virus is uncommon, and hence their recovery from the body is usually accepted as indicating a role in disease. With the viruses that infect the cells lining the gut, however, such carriage, particularly in children, is more common. The presence of virus in the faeces is not always associated with disease either in the gut or elsewhere in the body. These stool viruses fall into three groups. Group I includes the viruses that can be recovered from stools by cell culture or inoculation into newborn mice but which rarely cause any overt gastrointestinal disease. Group II includes viruses that cannot be grown routinely in cell cultures or in mice but which are associated with diarrhoea. Group III comprises the bacteriophages, which are viruses parasitic on the bacterial flora of the gut.

Diseases caused by the viruses of group I include poliomyelitis, aseptic meningitis, hand, foot and mouth disease, pleurodynia, myocarditis, and respiratory tract infections. The patients affected may be of any age but there is usually a predominance of children and young adults. Children may be found to excrete enteroviruses, often without evidence of disease, and this is common in areas of overcrowding and where there is poor sanitation and hygiene.

Diarrhoea is common in infants throughout the world and may often be infective in origin. Such diarrhoea is usually self-limiting with a peak incidence under the age of 2 years. Spontaneous recovery

occurs normally but a proportion of infants become sufficiently dehydrated to require medical attention with active restoration of fluid balance. Without this attention the mortality, particularly in the tropics, is high. The majority of cases are in young children but occasionally older children and adults may be affected. Common-source outbreaks ('winter vomiting', 'summer vomiting', some forms of food poisoning) also involve older children and adults. Bacterial causes do not account for more than about one-third of the total, and viruses have for long been presumed to be implicated in the remaining two-thirds.

Traditional cell culture methods led to the isolation and identification of viruses in stools but these viruses were not regularly implicated in cases of diarrhoea, and only recently, with the use of direct electron microscopy on stool extracts, have other viruses been detected in association with diarrhoea.

The list of these newly identified viruses has grown rapidly, and a paradoxical situation has emerged. On the one hand are viruses that can be grown from stools but which cause disease, if at all, elsewhere in the body. On the other are viruses that may cause disease in the gastrointestinal tract itself but which cannot be grown in routine cell cultures or in fetal gut organ culture, with the exception of some coronaviruses. All these viruses, however, are detected in stools by one means or another and replicate in the cells lining the gut.

The viruses

The viruses of groups I to III are listed in the Table and are illustrated in Figures 1 and 2.

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Table *Viruses found in stools*

Group	No. of serotypes
I Viruses that can be grown and passaged	
(a) Enterovirus	
Polio types 1-3	3
Coxsackie A types 1-22, 24	23
Coxsackie B types 1-6	6
Echo types 1-9, 11-27, 29-34	32
Enterovirus types 68-71	4
(b) Adenovirus types 1-33*	33
(c) Reovirus types 1-3	3
	Total 104
II Viruses detected by electron microscopy	
(a) Norwalk types 1-3†	3
(b) Rotavirus types 1-4?	4?
(c) Adenovirus	?
(d) Astrovirus	?
(e) Coronavirus	?
(f) Calicivirus	?
(g) SRVs‡	?
III Bacteriophages	
(a) Isometric (eg ϕ X 174)	Multiple
(b) Tailed	Multiple

*Not all adenovirus types have been reported as faecal isolates.

†Assuming Norwalk, Montgomery County, and Hawaii to be different.

‡A heterogeneous group including the following: W agent, Paver agent, Appleton and Higgin's agent, Ditchling agent, cockle virus.

Group I comprises those viruses discovered during the period 1950-70 by culture methods in cells or newborn mice. It now includes 68 enteroviruses, 33 adenoviruses, and 3 reoviruses, all of which, though found in the gut, are infrequently implicated as causes of gastrointestinal disease. Occasional common-source outbreaks of diarrhoea have been described, for example with echovirus type 18 (Eichenwald *et al.*, 1958), but these have usually involved closed groups of children, and it is not clear whether the virus caused the diarrhoea or took the opportunity to spread rapidly in a circumscribed susceptible population. Disease is associated with most of these group I viruses, but the lesions occur elsewhere in the body—in the central nervous system, muscle and skin with the enteroviruses and in the upper respiratory tract with both enteroviruses and adenoviruses.

Extensive studies using cell culture methods failed to implicate these viruses of group I as common causes of diarrhoea, particularly in babies, and the absence of demonstrable bacterial pathogens in more than 60% of patients with diarrhoea suggested that some other, then unknown, viruses might be involved. Since 1970 electron microscopy has been used to examine specimens of faeces directly, and this has led to the identification of the viruses of group II.

In this group are all the viruses detected by direct electron microscopy (EM) of faeces. They include Norwalk agent and the related Montgomery County and Hawaii agents, rotaviruses, astroviruses, coronaviruses, caliciviruses, and a variety of small, round

virus-like objects (SRVs) ranging in size from 20 to 35 nm.

The SRVs form a heterogeneous group, which vary in size and appearance, some showing a smooth outline in the EM and others a rough outline. Among them are several that have been associated with community outbreaks of diarrhoea, such as the W agent (Clarke *et al.*, 1972), cockle virus (Appleton and Pereira, 1977), and the Ditchling agent (Appleton *et al.*, 1977). It is probable that hepatitis A virus is also an SRV (Feinstone *et al.*, 1973). Solitary SRV particles are difficult to distinguish from the background debris in stools but it becomes progressively easier as the number of particles increases. For the same reason they are easier to recognise in a number of stool specimens from a common-source outbreak; most such outbreaks of non-bacterial diarrhoea have been associated with SRVs rather than with the other stool viruses. However, they are often found in stools from patients not involved in an outbreak, and it is important to note their occurrence in these non-outbreak situations, recording their size and appearance photographically until further tests to separate them into different groups are available to establish their role in disease.

The viruses of group II do not produce new infectious virus in routine cell cultures. Had they done so, this would have suggested that they were new viruses. As it is, while confirming previous negative culture results, there is nothing to suggest that they have not been current for some time. Some have been shown to replicate to some extent in particular cells (rotavirus in pig kidney, IB-RS-2 (Banatvala *et al.*, 1975), continuous monkey kidney, LLC-MK2 (Bryden *et al.*, 1977), and human embryo kidney (Wyatt *et al.*, 1976); astroviruses in human embryo kidney (Kurtz *et al.*, 1977); coronaviruses in embryo gut organ culture (Caul and Clarke, 1975)). Only Wyatt and his colleagues achieved serial passage of rotaviruses, and their results have still to be confirmed. Electron microscopy has also detected adenoviruses (commonly) and reoviruses (rarely) in faeces.

The bacteriophages of group III do not infect human cells and are included here only for the sake of completeness. They have not been implicated in disease and will not be discussed further. However, it should be remembered that any virus-like object seen by electron microscopy in the faeces may be of bacterial rather than human origin.

In addition to these virus particles seen in stools, viruses or viral antigens have been detected in intestinal cells in multiple sclerosis (Prasad *et al.*, 1977) and in motor neurone disease (Pertschuk *et al.*, 1977). The significance of these findings has still to be established but no gastrointestinal symptoms have been associated with them. There have been a

Fig. 1 The viruses found in stools. (a) Rotaviruses from stool. Note complete outer membrane in the majority of particles. (b) Reovirus type 3 from cell culture. Note absence of outer membrane on any particle. (c) Adenoviruses from stool, untyped and failed to grow in cell culture. (d) Adenovirus type 3 from stool, cultured in cells and indistinguishable from (c). (e) Astroviruses from stool. Note smooth outline and absence of central hollow on the particles. (f) Caliciviruses from stool. Note slightly larger size compared with the astroviruses, central hollow on five of the particles, and Star-of-David on the uppermost particle. (g) Norwalk agent mixed with acute-phase serum (reproduced, with permission, from Kapikian et al. (1972). All viruses stained with 3% potassium phosphotungstate, pH 7.0, and printed at a final magnification of $200\,000\times$. Scale bar = 100 nm.

Fig. 2 The viruses found in stools. (a) Enteroviruses (echovirus type 5) from cell culture. All enteroviruses have identical morphology and cannot be distinguished from each other in the electron microscope. (b) Small round viruses (SRVs) from stool, approximately 25 nm in diameter with a smooth outline. (c) SRVs from stool, approximately 30 nm in diameter with a 'feathery' outline. (d) Coronavirus from stool. Note long surface projections with spherical knob at end. (e) Tailed bacteriophages from stool. (f) Bacteriophage ϕX 174 from lysed culture of *E. coli*. (e) and (f) represent two types of bacteriophages likely to be present in stools, and the ϕX 174 would be classed as an SRV. All viruses stained with 3% potassium phosphotungstate, pH 7.0, and printed at a final magnification of 200 000 $\times$. Scale bar = 100 nm.

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number of reports of the recovery of virus from Crohn's disease and similar disorders (Cave *et al.*, 1973; Aronson *et al.*, 1975; Gitnick *et al.*, 1976), but details of the agent(s) are scanty. Whorwell *et al.* (1977) have observed rotaviruses in some cases of Crohn's disease, and this finding of a virus commonly detected in stools and found in association with intestinal lesions draws attention to the difficulties of proving that it contributes to the causation of a chronic disease.

Methods of detection

The viruses of group I (enteroviruses, reoviruses, and those adenoviruses that can be grown) are detected by conventional cell culture techniques in secondary monkey kidney cells, primary human amnion or fetal cells (especially kidney), or in newborn mice. Any virus grown is identified using specific antisera (Grist *et al.*, 1979).

All the viruses of group II were detected initially by electron microscopy, and this is the only method that can detect all the various morphological types of virus that may be present, provided there is enough to reach a detectable level. At least 10^6 particles per gram of faeces is necessary before virus can be detected, and this insensitivity of the microscope means that substantial amounts of virus in faeces may be missed. Nevertheless the titres of virus are often well above the threshold of detection. It is probable that the list of identified viruses is still incomplete but with other methods depending on the use of specific antibody to recognise the presence of virus, the electron microscope, though unsuited to screening large numbers, will retain its place as an essential tool in the investigation of infantile diarrhoea. The disadvantages of the electron microscope (its insensitivity, cost, and unsuitability for screening) have encouraged a search for alternative methods of virus detection. Most of these have been developed to detect rotavirus, and, unless specified, all the techniques listed below have been used in detecting this virus alone. They could be applied to other viruses when the technical details have been worked out and appropriate antisera prepared.

Partial growth of the virus in cell cultures has already been referred to, and both Banatvala *et al.* (1975) and Bryden *et al.* (1977) recommend infection of the cultures by centrifugation of the stools extract onto the cells. Since new infectious virus is not produced, new viral antigen appearing in the cells is detected by fluorescent antibody. Other methods to detect virus or viral antigen direct from stools include immunoelectrophoresis (Middleton *et al.*, 1976), radioimmunoassay (Kalica *et al.*, 1977), enzyme-linked immunosorbent assay

(ELISA) (Yolken *et al.*, 1977a), direct fluorescent antibody staining (Yolken *et al.*, 1977b), and complement fixation (Zissis *et al.*, 1978). A haemagglutinin has been described in the related calf rotavirus (Spence *et al.*, 1976) but has yet to be reported with human strains.

Two recent reports (Zissis and Lambert, 1978; Thouless *et al.*, 1978) show that there may be more than one serotype of human rotavirus. There may be multiple serotypes of other viruses as well, and this will cause problems in any test using antibody to detect the virus unless the predominant antigen is a group one.

Immune electron microscopy has been used to detect Norwalk agent (Kapikian *et al.*, 1972) but, in employing both antibody and electron microscopy, it can be said to have the disadvantages of both and, if crude stool extracts are used, is likely to have too many practical problems for routine use. This technique has been used to identify other viruses, such as hepatitis A (Feinstone *et al.*, 1973), using convalescent serum. Since other antibodies will be present in human sera this approach must be very carefully controlled for the results to be meaningful, and it may not work with sera from babies too young to be fully competent immunologically.

Infection and disease

In some virus diseases, such as measles and chickenpox, a high proportion of those infected develop symptoms. If infection with such a virus can be demonstrated in a patient with typical symptoms it is reasonable to assume a causal relationship. No such assumption may be made with any of the viruses found in the gut, particularly in children. Enteroviral excretion with no evidence of illness is often found in young children (Bell *et al.*, 1961; Patterson and Bell, 1963), and this has to be borne in mind when considering the role of other viruses found in the gut. In the case of rotaviruses, morphologically similar viruses cause diarrhoea in the young of other species and, since this can be verified experimentally in gnotobiotic animals, their role in causation has been established. The human virus, too, has been shown to cause diarrhoea in gnotobiotic animals (Middleton *et al.*, 1975; Mebus *et al.*, 1977; Snodgrass *et al.*, 1977b). In man, however, this straightforward cause-and-effect relationship is harder to establish. Excretion without diarrhoea in newborn animals is rare and, in most species, young animals rapidly become refractory to the disease with age. Where excretion without overt disease has been found in man (Chrystie *et al.*, 1975; Murphy *et al.*, 1977; Madeley *et al.*, 1978) it has usually been in newborns, and disease associated with virus occurs in older children with a peak in the

1-3 year age group, as Elias (1977) has shown with antibody studies. Though attempts to infect adults deliberately have not proved very successful (Middleton *et al.*, 1974), there have been reports of natural adult infection (Ørstavik *et al.*, 1976; von Bonsdorff *et al.*, 1976, 1978; Meurman and Laine, 1977), and it has been suggested that some infants in hospital are infected by adults (von Bonsdorff *et al.*, 1976). Since not all babies will be equally susceptible owing to variations in immunity, type of feeding, general home circumstances, and variation between individuals, the situation in man is clearly complex, and this applies to all the other viruses observed in faeces. Unpublished observations in Glasgow have shown that all viruses may be seen in healthy babies as well as in those with diarrhoea. Rotaviruses have been observed in 208 patients up to June 1977, and, of these, 161 (77%) were associated with diarrhoea. Another 21 (10%) had a more doubtful association (previous negative stools, second virus observed, etc) and the remaining 26 (13%) were from normal babies or from those with no diarrhoea. Comparable figures for astroviruses were 62 (80%), 6 (8%), and 11 (12%) and for caliciviruses 15 (53%), 6 (21%), and 7 (26%).

It is common to find more than one virus at a time in babies' stools and it is then impossible to say which virus, if any, is the prime cause of any disease. Rotaviruses have been found throughout the world and most commonly in the stools of babies with diarrhoea. This suggests that they usually cause some disruption to gut function, though the extent to which they may be denizens of hospitals, infecting babies soon after admission, needs to be investigated. Ward cross-infection might explain the wide variation in positivity rates in babies with diarrhoea in hospital, ranging from 57% (Middleton *et al.*, 1977) to less than 20% (White *et al.*, 1974; Schoub *et al.*, 1975).

Mechanisms of infection

The gut is well designed for the growth of micro-organisms. For viruses there is a constant renewal of cells in various stages of differentiation at a steady temperature and, though there may be some hostile elements in the environment (acids, bile salts, antibodies, enzymes, etc) the viruses commonly found in the stool show resistance to physical or chemical inactivation.

Little direct information is available about the processes of infection with any human virus *in vivo*. Most of it comes from animal models, which do not necessarily mimic every detail of infection in man. Pathways of infection have been worked out in chimpanzees fed oral polio vaccine but these experiments did not show which cells or what proportion of

them were initially infected. There is evidence from *in vitro* studies that there are specific receptor sites on susceptible cells for several enteroviruses. Whether these receptors operate *in vivo* and decide which cells become infected is not known. If they do, the availability of receptor sites could be an important factor in fixing the limits of an infection.

With the possible exception of adenoviruses, it is rare for the amount of virus produced in the stools by infections with the group I viruses to reach the levels detectable by electron microscopy. In contrast, the titre of virus resulting from rotavirus astrovirus, calicivirus, and other EM-detectable infections is frequently very much higher, reaching titres of 10^{11} /g faeces or more. This might be expected to reflect a more widespread involvement of the intestinal cells and hence more severe symptoms. However, the titre of virus observed does not always parallel the severity of the disease, and there may be several reasons for this. It is difficult to define a 'standard stool' and then to ensure that the extracts examined under the microscope are all equally representative of virus content of the original stool. Even if they are, the amount of virus may not reflect the extent of functional damage to the gut cells.

Different viruses may infect different cell types and therefore produce disease through different mechanisms. One virus may infect a large number of cells superficially, producing a large amount of virus and little functional upset, while another may infect an important minority cell type only and cause considerable upset. Poliovirus rapidly kills cells in culture but does not cause diarrhoea *in vivo*, suggesting that only a few cells or no important cells are infected in the gut. Rotaviruses are found in the epithelial cells of the duodenal villi (Bishop *et al.*, 1973), and in experimental animals viral antigen detected by immunofluorescence is maximal in the cells towards the tip of the villus (Snodgrass *et al.*, 1977a). These are the most differentiated cells with a variety of potential functions, not all of which will be expressed at the same time but they will be influenced by the type of feeding the baby receives. An infecting virus damaging only one kind of cell may thus vary in its effect, depending on which functions are being expressed at that time.

Holmes *et al.* (1976) have suggested that lactase is both a receptor for rotaviruses and an uncoating enzyme for the virus. This would make intestinal lactase a promoter of infection and possession of it a liability; and newborns, who secrete relatively more lactase than older children, would be likely to be more susceptible to the virus. In fact, they appear to be less susceptible than children 6 months to 2 years older, and consequently this theory does not appear to be convincing. In contrast, Agus *et al.* (1973) have

shown that considerable depression in the enzymic activities in human gut biopsy specimens follows experimental infection with the Norwalk agent, reducing the chance of infection continuing if any of these enzymes are important to the replication of the virus. Nevertheless, in the interplay between virus and cells, the nature of the baby's food and the enzymes it induces may be important in deciding whether an incoming virus becomes established in the cells of the gut.

Breast-fed babies are commonly thought to be less likely than bottle-fed babies to suffer from infective diarrhoea, and this has been ascribed to the presence of maternal secretory antibody in the milk. Anti-rotavirus factors have been found in human milk (Matthews *et al.*, 1976; Thouless *et al.*, 1977; Simhon and Mata, 1978) though whether they are true antibodies is not yet certain. Nevertheless detectable levels of rotaviruses have been found in the stools of breast-fed babies (Totterdell *et al.*, 1976) despite the widespread experience of rotaviruses, reported by Kapikian *et al.* (1975), Elias (1977), and others, which makes it likely that the majority of mothers will have had a prior infection at some time in their lives. Passive antibody may therefore prevent overt disease without preventing infection, as has been found in animal experiments (Snodgrass *et al.*, 1977b). The role of the baby's own secretory antibodies is even less certain but there is no suggestion so far that symptomless excretion is due to IgA antibody coating the virus. Such virus usually appears normal in the EM (Madeley *et al.*, 1978) but this does not rule out the presence of small amounts of avid neutralising antibody on the virus surface. Watanabe and Holmes (1977) suggest that such small amounts are usually present and could account for non-growth of rotavirus and others in cell culture, although this does not explain why a similar inhibition is not found with the enteroviruses, adenoviruses, and reoviruses that can be grown from stools.

Where the infecting virus is so readily accessible to antibody, passive immunisation might be used to prevent disease though its use would probably have to be restricted to specific situations of high risk. There is evidence from animal experiments that gammaglobulin given orally may give such protection (Snodgrass and Wells, 1976; Snodgrass *et al.*, 1977b).

Adenoviruses

The relation of the viruses found in stools to disease is complex and is full of paradoxes. Several of them are epitomised by the adenoviruses. Previous studies, for example the Virus Watch Program (Fox *et al.*, 1969), have found that adenoviruses may be excreted

in stools for prolonged periods by young children without evidence of disease. Their results were obtained using cell cultures, but similar results have been obtained by electron microscopy (Scott *et al.*, 1977, unpublished observations) with the same kind of study group. It is not difficult to grow adenoviruses from stool specimens, and occasionally more than one serotype is isolated from a single stool (Bell, 1978, unpublished observations). Surprisingly, only a few of the adenoviruses seen by electron microscopy can be grown in cell cultures although why some stool adenoviruses can be grown and others cannot is a mystery. The non-growers may be serotypes whose characteristics (other than possessing typical adenovirus morphology) are unknown, or familiar ones that fail to grow for some unknown reason. Attempts to type them by immune electron microscopy have not given clearcut results (Flewett *et al.*, 1975) but there was no suggestion that they were new serotypes. They are found in 5-8% of the stools of infants (Davidson *et al.*, 1975; White and Stancliffe, 1975; Madeley, 1978, unpublished observations), but occasionally appear to be involved in outbreaks of diarrhoea (Flewett *et al.*, 1975; Tufvesson and Johnsson, 1976; Whitelaw *et al.*, 1977). Their presence appears to be an incidental finding. The extent to which this is true of other viruses remains to be established.

Future progress

The role of the group I viruses in disease is now largely established although there are still gaps in our knowledge. The part played by enteroviruses in heart and motor neurone disease is still under investigation, as are the mechanisms of the failure of oral polio immunisation in underdeveloped countries. Although reoviruses have been known for over 20 years, they have still to be linked definitely with any disease syndrome. In infantile diarrhoea, much also remains to be done before the relation of viruses to disease is fully understood despite some optimistic assertions. In this situation what can the diagnostic laboratory do?

The answer to this question depends on the resources and interests of the laboratory. Routine examination of stools from babies with diarrhoea by electron microscopy will provide a record of the viruses present in each stool examined which, though valuable in recording what viruses are present in the community, will not be a proper investigation into the disease of infantile diarrhoea. Examination of the stools from different groups of patients and from each patient on several occasions, both with and without symptoms, is necessary for this. Such an investigation requires a very much greater commit-

ment of both time and interest than the normal routine operation of a laboratory can provide. In the absence of an electron microscope, which remains the only catch-all method, the aims must be more circumscribed still. Any investigation must then be confined to the viruses for which both antisera and tests are available and, while it can be argued that to concentrate on rotaviruses alone will not result in much of significance being missed, this argument overlooks the growing evidence of the involvement of other viruses in endemic cases of diarrhoea (for example, Madeley *et al.*, 1977; Middleton *et al.*, 1977) and, even more so, in epidemics.

Perhaps more important still, a concentration on one virus to the exclusion of others makes it difficult to answer some of the basic questions about infantile diarrhoea to which answers are still needed:

How are stool viruses transmitted to both newborns and older babies? (The mechanism may not be the same for both.)

What, if any, is the role of adults in transmission?

Why are some babies infected and not others?

What constitutes an infection severe enough to cause diarrhoea?

What is the significance of multiple virus infections, simultaneously or sequentially, and do different viruses act in synergy in the causation of diarrhoea? Are there 'diarrhoeal' babies (those who are inherently more liable to develop loose stools)?

These are some of the unanswered questions; readers will have little difficulty in adding to the list. Some of the answers will come from careful epidemiological studies in which many laboratories can and should take part. In so doing the staff will have to assess the commitment that they can make with the facilities available to them, and should also be careful not to deceive themselves as to the extent of their own investigations. Owing to the limitations on the extent to which experiments can be done on babies, the answers will take time to find.

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