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Classical and molecular techniques for the diagnosis of viral gastroenteritis¹

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Diarrhoeal diseases are very frequently caused by infections, particularly in children under 5 years of age. Globally, the annual mortality is 3.3 million (or approximately 4 per 1000) in this age group (Bern et al., 1992). The median incidence of diarrhoea per child per year is 2.3–3.9 episodes in Africa, Asia and Latin America, amounting to 1.1–1.7 billion episodes of diarrhoea worldwide in the 500 million children under 5 years old (Bern and Glass, 1994).

Diarrhoea is caused by very different agents (viruses, bacteria, parasites, toxins), with bacterial infections being of relative higher importance in developing countries and virus infections in developed countries. In both settings, however, group A rotaviruses are the most frequent cause of severe childhood diarrhoea causing an estimated 870 000 deaths per year (Institute of Medicine, 1986). It is remarkable that despite advanced techniques, 30–50% of diarrhoeas are not associated with a known pathogen.

Among the viral causes of childhood diarrhoea episodes in developed countries the median detection rates (range) were as follows (Bern and Glass, 1994):

- Rotaviruses 32% (11–68%)

- Enteric adenoviruses 6% (0.9–10%)
- Small round structured viruses (caliciviruses) 3.9% (1–13%)
- Astroviruses 3.6% (1–5%)

Most of these viruses are endemic, and children become infected at an early stage with any of these. However, many viruses have also been identified as agents causing epidemic outbreaks in day care centres, old people's homes, schools, or the general adult population (Norwalk viruses, group A–C rotaviruses, caliciviruses and astroviruses).

In the following the main viral causes of gastroenteritis will be briefly described and the main techniques to achieve their diagnosis will be discussed.

1. Rotaviruses

Rotaviruses are the major cause of gastroenteritis in infants and young children and in a vast variety of animal species. Rotaviruses are a genus of the Reoviridae family and as such have a genome of double stranded RNA consisting of 11 segments which can be easily separated by polyacrylamide gel electrophoresis. The genomic segments code for six structural (VP1–VP7) and five non-structural proteins (NSP1–NSP5). The structural proteins are located in the core (VP1–VP3),

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inner shell (VP6) and outer shell (VP4, VP7) (Mattion et al., 1994)

A classification scheme of rotaviruses has been derived from immunological reactivities of various parts of the particles and from genomic composition. Groups A–E can be differentiated according to the lack of serological cross-reactivities of the inner capsid protein VP6. Within group A, rotaviruses subgroups are defined (I; II; I + II; nonI, nonII; epitopes also on VP6) according to exclusive reactivities of two monoclonal antibodies. Types within group A are determined by cross-neutralization studies as *serotypes* or by sequence comparison as *genotypes* (typing in other groups is rudimentary). As there are two surface proteins carrying neutralization-specific antigens (VP4 and VP7), a double classification similar to that developed for influenza viruses has been established, differentiating G types (for VP7 which is a glycoprotein) and P types (for VP4 which is a protease-sensitive protein, being posttranslationally cleaved into its subunits VP5* and VP8*). So far, 14 G types and more than 20 P types have been differentiated indicating extensive genomic diversity within group A rotaviruses (Estes, 1996). As these proteins are coded for by different RNA segments and as rotaviruses reassort readily in doubly infected cells both in vitro (Garbarg-Chenon et al., 1984) and in vivo (Gombold and Ramig, 1986; Ward et al., 1990), the observed diversity due to various combinations of VP7 and VP4 types becomes very large.

Rotaviruses infect the small intestine after oral ingestion and spread via the faeco-oral route. Virus multiplication is in the mature epithelial cells at the tips of the villi of the small intestine. Cell death and desquamation lead to reduction of adsorption and digestion (primary malabsorption) and to villous atrophy. There is a reactive crypt cell hyperplasia which is accompanied by increased secretion thought to contribute to the severity of diarrhoea. The number of virus particles in the gut at the peak of the diarrhoea can be as high at 10^{11} /ml of faeces. Diagnosis is therefore relatively easy using electron microscopy (EM), ELISA or passive particle agglutination techniques.

Electron Microscopy is an important tool to detect viral gastrointestinal infections. Particles with morphologies typifying rotaviruses, adenoviruses, caliciviruses, small round structured viruses (SRSVs), astroviruses, small round viruses (SRVs: enteroviruses and parvoviruses), coronaviruses and toroviruses can be readily identified (Doane, 1994). As ELISA-based serological tests are not routinely available for all these viruses associated with diarrhoea, EM is widely used for rapid diagnosis. Although it is very cheap in consumables, it requires considerable time from staff with special expertise (Madeley, 1995).

Serological assays to detect rotaviruses are in wide use. Mostly direct or indirect enzyme-linked immunosorbent assays (ELISAs) are used: broadly cross-reactive group A rotavirus antibodies, adherent to the well of a microtitre plate, are reacted with a 10% faecal suspension followed by a washing step; bound rotavirus is then detected with either enzyme-labelled antirotavirus antibody followed by reaction with a chromogenic substrate (direct) or with unlabelled antirotavirus antibody followed by incubation with a second anti-species IgG antibody tagged with an enzyme and then incubated with substrate (indirect). The tests can be calibrated and quantified. Various enzyme-substrate systems are in use. If the detecting antirotavirus antibody is type-specific, the test can be used for G and P typing; this however, depends on the presence of double-shelled virus particles in the clinical specimen. Another frequently used rapid technique is a passive particulate agglutination test (Ruggeri et al., 1992). Details of procedures can be obtained from Yolken and Wilde (1994), and Gray and Wreghitt (1995).

The viral genome can easily be detected after phenol extraction of RNA from crude or semipurified rotavirus-containing specimens and separation by polyacrylamide gel electrophoresis (PAGE) followed by silver staining (e.g. Follett et al., 1984). More recently, more sensitive techniques to detect and analyze rotavirus genomes have been developed. Using rotavirus-specific primers in a reverse transcription (RT)-polymerase chain reaction (PCR) (Simmonds, 1995) has not only allowed sensitive detection but also typing for both G and P types (Gouvea et al., 1990a; Gentsch et al., 1992).

Table 1
Techniques used to diagnose infections with viruses causing gastroenteritis

Technique	Sensitivity (particles/ml)	Specificity	Labour requirements	Costs
Electron microscopy				
a) Direct	10 ⁶	Low	High	Medium
b) Immune	10 ⁴	High	High	Medium
Passive particle agglutination	10 ⁶	Medium	Low	Low
ELISA	10 ⁵	Medium	Low	Low
Hybridization (probes)	10 ⁵	High	High	Medium
PCR/RT/PCR	10 ²	High	High	High
Cell culture	10 ²	Low	High	High

Rotavirus genomes can also be detected by hybridizing radiolabelled biotinylated probes to Northern blots of viral RNA. Hybridized probes are visualized by autoradiography or reaction with enzyme-coupled streptavidin and substrate, respectively (Fernandez et al., 1992). However, for diagnostic purposes the ELISA which is of similar sensitivity but much easier to perform, is mostly preferred.

Human rotaviruses can be grown *in vitro* on monkey kidney cells in the presence of trypsin (Ward et al., 1984), but the procedure is not in routine use in most diagnostic laboratories.

Table 1 gives an overview of relative sensitivity, specificity, labour requirements and costs involved in the different procedures discussed above. From this it is apparent that EM is important for practical purposes as visualizing all viruses, but is insensitive. Relatively high specificity can be achieved by immune EM (IEM; see below). RT-PCR is very sensitive and specific (depending on primers used), but is relatively laborious and prone to errors (false positive results due to contamination, and false negative results due to enzyme inhibitors in nucleic acid extracts of chemical specimens). ELISAs are of medium sensitivity, most apt for processing large numbers of specimens and of relatively low costs.

After a short incubation period of 24–48 h, the onset of illness is sudden with watery diarrhoea, vomiting and rapid dehydration. Rotavirus infection is a major cause of infantile death in the developing world if untreated. It should be noted, though, that the clinical symptoms of rotavirus

infections may vary widely, and asymptomatic infection of neonates with so-called nursery strains has been described (Hoshino et al., 1985).

Treatment is by oral, subcutaneous or intravenous rehydration. There is a WHO agreed formula of oral rehydration salts (ORS) to which glucose is added (Pierce and Hirshhorn, 1977).

After neonatal or primary rotavirus infection, a mainly serotype-specific humoral immune response is elicited, but there is also partial protection against subsequent heterotypic infections (Bishop et al., 1983; Chiba et al., 1986; Friedman et al., 1988; Linhares et al., 1989). The exact correlates of protection are not known, but the levels of coproantibodies of the IgA subclass are the best (Corthier and Franz, 1981; Coulson et al., 1992; Matson et al., 1993; Offit et al., 1993). The exact role of rotavirus-specific cytotoxic T-cell (CTC) responses (Offit et al., 1993) remains to be determined.

The epidemiology of rotavirus group A infections in man is complex: at any one time and site there is co-circulation of rotaviruses of different G types, types G1–G4 representing 95% of co-circulating strains worldwide and type G1 approximately 50% (Flores et al., 1988; Desselberger, 1989; Beards et al., 1989; Gouvea et al., 1990b; Matson et al., 1990; Padilla-Noriega et al., 1990; Noel et al., 1991). In one country, different serotypes can show regional differences (Noel et al., 1991). Group B rotaviruses have caused widespread outbreaks in children and young adults in China (Hung, 1988; Fang et al., 1989), but apparently not elsewhere.

Vaccine development to protect against rotavirus infections has been ongoing since the early 1980s and has been reviewed by Desselberger (1993), and more comprehensively by Kapikian (1994) and Vesikari (1994). Due to the enormous genomic and antigenic diversity of rotaviruses, results of vaccine trials have been mixed. In most trials animal rotaviruses (of bovine or simian origin) have been used as live attenuated virus vaccines. Protection from infection and mild disease was only modest (and best when the vaccine serotype was related to that of the predominant circulating human wildtype rotavirus strain), whereas partial protection *preventing severe disease* was achieved in most cases, possibly due to cross-reactive coproantibody (IgA) or CTC responses (Rennels et al., 1996). The vaccine response is widened by applying a cocktail of viruses, e.g. containing a native rhesus rotavirus (RRV) of G3 type and reassortants carrying the VP7 genes of human serotypes G1, G2 and G4 in the RRV genetic background (Rennels et al., 1996). In the USA, cocktail vaccines have been submitted for FDA approval which is being considered at present.

2. Adenoviruses

Human adenoviruses occur in 47 serotypes and six subgenera (A–F; Hierholzer et al., 1988). Most of these are involved in infections of lymphoid tissues and the respiratory tract. Although many of them can be detected in faeces without diarrhoea, adenoviruses of subgenus F (types ad40 and ad41) were found to be exclusively associated with diarrhoea (Johansson et al., 1980). Adenoviruses of subgenus A (types 12, 18 and 31) can also be associated with diarrhoea, particularly in children and in immunocompromised adults (Hierholzer, 1992). Enteric adenoviruses are fastidious but grow in the 293 cell line (Takiff et al., 1981). Within ad40 and ad41 types, different genotypes have been differentiated by extracting viral DNA, producing restriction endonuclease fragments and separating them by PAGE followed by ethidium bromide staining or, when radiolabelled, by autoradiography (Kidd et

al., 1984; van der Avoort et al., 1989). The diagnosis is by EM and immune EM, ELISA, latex agglutination test or PCR (Herrmann et al., 1987; Grandien et al., 1987; Allard et al., 1990).

Diarrhoea is a preponderant symptom which usually lasts for 8–12 days; fever and vomiting are mild (Uhnoo et al., 1984). Neutralizing antibodies are found in one third to one half of children investigated (Kidd et al., 1983).

3. Norwalk and Norwalk-like viruses

These small viruses of 27 nm diameter and the characteristic appearance as SRSV by EM are the cause of numerous outbreaks of acute gastroenteritis worldwide ('winter vomiting'); the names of the viruses are mostly derived from the sites of the outbreak. As none of the human viruses grow in tissue culture, IEM was crucial for the classification of these viruses. In this technique, viral suspensions are mixed with convalescent sera and the virus-antibody complexes detected by EM. Antibody can also be coated onto the grids before reaction with the faecal specimen (solid phase immune EM, SPIEM; Lewis et al., 1988). To date, four serotypes are distinguished (Lewis, 1991):

- (1) Norwalk virus (NV)
- (2) Hawaii Agent (HWA)
- (3) Snow Mountain agent (SMA)
- (4) Taunton agent (TA)

Viruses isolated in Japan (Sapporo, Osaka) seem in part to differ (Okada et al., 1990).

ELISAs for Norwalk-like viruses have been developed (Herrmann et al., 1985; Madore et al., 1986; Treanor et al., 1988).

Significant progress has been made with the molecular cloning and sequencing of Norwalk virus (Jiang et al., 1990, 1993b) which showed it to be a calicivirus by genome organization and expression. Similarly, by genome analysis, Southampton virus was found to be a calicivirus (Lambden et al., 1993). These viruses possess a single stranded RNA genome of positive polarity of approximately 7.6–7.7 kb size. Complementary DNA containing open reading frame 2 (ORF2) was expressed from baculovirus recombinants in insect cells and produced large amounts of the

structural protein forming virus-like particles (VLPs, Jiang et al., 1992a). These have been used as antigens in ELISA, and it was shown that NV or related viruses infect children worldwide in large numbers, and 60–80% of young adults have NV-reactive antibody (Gray et al., 1993). The availability of limitless amounts of purified recombinant antigen has put the seroepidemiology of Norwalk-like viruses on a new footing.

From available sequences, primers for RT-PCR have been chosen which allow amplification of most of the genomes of SRSVs detected by EM/IEM (Ando et al., 1995). PCR amplification products from various isolates around the world showed co-circulation of different genotypes/serotypes in different countries (Wang et al., 1994; Ando et al., 1995).

Diagnosis is at present preferentially by EM as ELISAs have not been fully developed yet due to the presence of serotypes. In addition, RT-PCRs are increasingly used (Jiang et al., 1992b; Atmar et al., 1993; Wang et al., 1994; Ando et al., 1995).

Norwalk-like viruses are transmitted by the faeco-oral route and were found to be causative agents of various outbreaks of viral gastroenteritis occurring in recreational camps, schools and nursing homes, on cruise ships, around contaminated swimming pools, or in the community (Greenberg et al., 1981).

The incubation period is less than 24 h, and the symptoms are characterized by nausea and very heavy vomiting, less frequently diarrhoea. The symptoms last for 1–2 days only, secondary cases are frequent.

Humoral (systemic and local) immunity develops, but there is no correlation between NV antibody titre and protection from reinfection as was shown in volunteer studies (Parrino et al., 1977; Gray et al., 1994). Reinfected volunteers, who become ill, mount a more vigorous and rapid immune response than individuals who have asymptomatic reinfections (Gray et al., 1994).

4. Caliciviruses

Caliciviruses, characterized by their unique structure in electron micrographs, have been

found to cause numerous outbreaks of gastroenteritis (epidemic vomiting, gastric flu) which clinically are indistinguishable from those caused by Norwalk like viruses (Cubitt, 1994). As is now evident (see previous section), all of these viruses are relatively closely related members of the Caliciviridae family (Cubitt et al., 1995). However, the sequence organization of a classical human calicivirus puts it in closer relationship to animal caliciviruses than to human SRSVs (Liu et al., 1995).

5. Astroviruses

Astroviruses have been recognized as aetiological agents for diarrhoea in infants since 1975 (Herrmann et al., 1991), but also cause diarrhoea in animals (sheep, pigs, cats, dogs, deers). There are at least seven serotypes which have originally been distinguished by IEM (Lee and Kurtz, 1994; Noel et al., 1995). Two of them have been fully sequenced and possess a genome of single stranded RNA of positive polarity of 7kb (Jiang et al., 1993a; Willcocks et al., 1994). In contrast to most of the other SRSVs, astroviruses grow well in tissue culture (CaCO₂ cells, LLCMK2 cells; Lee and Kurtz, 1981; Willcocks et al., 1990). Astrovirus type- and serotype specific ELISAs have been developed (Herrmann et al., 1988, 1990, 1991), and RT PCR (of part of ORF2 of the genome, coding for capsid protein) followed by sequencing yielded genotypes which are very well correlated with the known serotypes determined by IEM or ELISA (Noel et al., 1995). Due to the presence of various serotypes, EM is still most important for establishing the diagnosis.

The infection normally occurs in childhood; two thirds of the 3–4-year old and 87% of the 5–10 year old children in England were found to carry specific antibody indicating previous infection (Kurtz and Lee, 1978). The incubation period is 3–4 days, and diarrhoea lasts 2–3 days. Large outbreaks of gastroenteritis due to astroviruses have been observed in the community (Utagawa et al., 1995).

6. Toroviruses and Coronaviruses

Those viruses, members of distinct families of positive stranded RNA viruses, are well recognized aetiological agents of diarrhoea in animals. However, their regular association with gastroenteritis in humans is controversial (Horzinek et al., 1987; Caul, 1994).

7. Human Immunodeficiency Virus (HIV)

HIV can infect the gut directly, most likely through macrophages in the submucosa, leading to chronic diarrhoea, aggravated by secondary infections (for review see Grunfield and Feingold, 1992). Weight loss and wasting occur by reduced energy intake leading to a negative energy balance (Macallan et al., 1995). Diagnosis of HIV infection is by serology. A more detailed discussion of HIV gut infections is beyond this review.

8. Outlook

Viruses are a major cause of acute gastroenteritis in infants and young children worldwide and cause widespread outbreaks of the disease. The aetiological agents are members of many different virus families, mostly RNA viruses. There is intense ongoing research on improving rapidity and specificity of the diagnosis and on the molecular epidemiology of these viruses.

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