

Diarrhoea Due to Viruses Other Than Rotaviruses

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INTRODUCTION

Gastroenteritis is among the most common illnesses affecting humans and has greatest impact at the extremes of age, severely affecting children and the elderly. The spectrum of disease can range from asymptomatic infections to severe disease with dehydration, which can be fatal. Prior to 1972, the aetiology of most episodes of gastroenteritis was unknown, and cases were attributed to a multitude of causes, including teething, weaning, diet, old age, drugs and malnutrition. Intensive investigation of enteric infections in the past three decades has resulted in the discovery of many new agents filling in the "diagnostic gap" in diarrhoeal disease. With the identification of the Norwalk virus in 1972 (Kapikian *et al.*, 1972), rotavirus in 1973 (Bishop *et al.*, 1973), astroviruses in 1975 (Madeley and Cosgrove 1975; Appleton *et al.*, 1975), enteric adenoviruses in 1975 (Wadell *et al.*, 1987) and other caliciviruses in 1978 and subsequently (Chiba *et al.*, 2000), it has become increasingly clear that viruses cause a significant proportion of the enteric illnesses that did not earlier have a defined aetiology (Glass *et al.*, 2001; Yamashita *et al.*, 1998).

Criteria to define a virus as an etiologic agent of gastroenteritis include (i) identification of the virus more frequently in subjects with diarrhoea than in controls, (ii) demonstration of an immune response to the specific agent, and (iii) demonstration that the beginning and end of the illness correspond to the onset and termination of virus shedding, respectively (Kilgore and Glass 1997). So far, these include human caliciviruses (HuCVs), rotaviruses, astroviruses and the enteric adenoviruses. In immuno-compromised patients, gastrointestinal cytomegalovirus infections also cause significant morbidity. Coronaviruses, toroviruses and picobirnaviruses have also been found to be associated with diarrhoea in some studies, but definitive data is not yet available (Holmes 2001). Similarly, in conditions such as HIV, it has been difficult to obtain definitive data on the role of enteric viruses in the causation of symptoms (Pollok 2001). The study of rotaviruses, enteric adenoviruses, and astroviruses has been facilitated greatly by the ability to propagate these viruses in cell culture, which has allowed the production of

reagents for use in diagnostic studies, a better understanding of factors correlated with immunity to infection, and the elucidation of each virus's life cycle. Although human caliciviruses have defied numerous attempts to propagate them in cell culture to date, recent developments in their study by using molecular biology techniques have increased our ability to diagnose and study infections due to these agents (Atmar and Estes 2001).

EPIDEMIOLOGY AND CLINICAL FEATURES OF VIRAL GASTROENTERITIS

Infections with gastroenteritis viruses differ from bacterial enteric infections in that they affect children in both developing and developed countries, suggesting that they may be transmitted by means unrelated to contaminated food or water. The two distinct patterns of viral gastroenteritis, endemic childhood diarrhoea and epidemic disease, reflect the differences in the pathogens, transmission and host response. These have a direct bearing on strategies for prevention and control (Table 1).

Table 1 *Epidemiologic patterns of viruses causing acute gastroenteritis*

	<i>Endemic childhood disease</i>	<i>Epidemic disease</i>
Viruses	Rotavirus (group A) Astrovirus	Caliciviruses (NLVs) Rotavirus (group B)
	Enteric adenoviruses Caliciviruses (NLVs & SLVs)	Astrovirus
Mode of transmission	Unknown; ? contact; fomites, droplets, aerosols or person-person	Food, water, ? contact, droplets, and aerosols
Reservoir	Humans	Humans
Antibody	High prevalence by 5 years of age	Seroconversion in epidemic
Immunity	Good	Short-term (calicivirus)
Virus variation	Limited discrete serotypes (except caliciviruses)	Many antigenic variants of caliciviruses
Public health control measures	Vaccine-RV (group A)	Outbreak control; improved food safety and handling

Childhood diarrhoea is best exemplified by the group A rotaviruses, but a similar pattern of infection and illness is seen with enteric adenoviruses, astroviruses and Sapporo-like viruses. These agents infect children during the first few years of life, with first infections being symptomatic and protecting against subsequent disease. Disease is caused by a limited number of specific serotypes and incidence decreases with increasing age.

The universal nature of these infections implies that improvements in sanitation and food quality are unlikely to decrease incidence of disease. In developing countries, children can have 5–10 episodes of gastroenteritis each year during their first 5 years of life, and although most episodes are mild, some can progress to dehydration, malnutrition and death (Bern *et al.*, 1992). In developing countries taken as a whole, one in 40 children dies of diarrhoea making this the second most common cause of death in this age group.

Caliciviruses

The human caliciviruses, particularly the “Norwalk-like viruses” (NLVs) are the best examples of viral agents causing epidemic disease. The Norwalk virus is the representative agent of a heterogeneous group of viruses, also called small round structured viruses (SRSVs), human caliciviruses (HuCVs) or the Norwalk-like family of agents. The antigenic interrelationships among the many members of this class are complex, and the agents are usually identified by the locale where an outbreak occurred (e.g., Hawaii, Snow Mountain, Montgomery County, Taunton, Amulree, Sapporo, and Otofuke). Levels of antibody specific to the Norwalk agent are low during childhood but reach 50% by middle age. In developing countries, antibodies are acquired at an earlier age; peak incidence of illness may also occur among younger age groups than in developed nations. The incubation period is 24–48 hours, and the mean duration of illness is 12–60 hours. Nausea is prominent, with vomiting, non-bloody diarrhoea, and abdominal cramps occurring in most cases. These symptoms are experienced by all age groups, but diarrhoea is relatively more prevalent among adults, whereas a higher proportion of children experience vomiting. From 25%–50% of affected persons also report headache, fever, chills, and myalgias. Adults have died during illness caused by Norwalk-like viruses, presumably from electrolyte imbalance. Late sequelae have not been reported, but the elderly often report persistence of constitutional symptoms for up to several weeks. Routes of transmission that have been documented include water, food (particularly shellfish and salads), aerosol, fomites, and person-to-person contact (Green *et al.*, 1998; Ohyama *et al.*, 1999; Shrier *et al.*, 2000). Infectivity can last for as long as 4 days after resolution of symptoms. Presymptomatic shedding has been suspected on epidemiologic grounds but not proven in volunteer studies.

Numerous reports have described the course of outbreaks caused by Norwalk-like agents, usually involving adults and older children. The settings are diverse and include banquets, cruise ships, geriatric facilities, psychiatric wards, emergency rooms, cafeterias, recreational lakes, swimming pools, campgrounds, football teams, hotels, schools, dormitories

fast food restaurants, and others (Becker *et al.*, 2000, Schaub and Oshiro, 2000 Glass *et al.*, 2001). Norwalk-like agents probably create a low background level of infection in a community until an infected individual contaminates a common source, and an explosive outbreak occurs. Although secondary cases can multiply the number of persons affected, outbreaks are generally limited to 1–2 weeks unless transmission is facilitated by a closed environment (e.g., a nursing home) or prolonged by renewal of the susceptible population (e.g., a new set of passengers on a cruise ship). It is also important to emphasize that in food-borne outbreaks, up to 68% do not have an identified cause, and infections due to agents such as HuCVS which are difficult to identify may be under-diagnosed (Mead *et al.*, 1999). Recent studies from the Netherlands using molecular detection techniques have shown that for cases of gastroenteritis presenting to a general practitioner approximately 5% were caused by NLVs and 2.4% by Sapporo-like viruses (SLVs), with NLVs affecting all age groups and SLVs mainly children (deWit *et al.*, 2001). However, for outbreak investigations, 80% of all outbreaks were caused by NLVs in the Netherlands (Koopmans *et al.*, 2001). In Finland, viruses were identified in 60% of all diarrhoeal episodes in children less than two years of age. Of these, 24% were rotaviruses, 19% caliciviruses, 4% rotaviruses and 4% adenoviruses (Pang *et al.*, 2000). The epidemiology of NLVs and SLVs is complex, with multiple circulating strains. Typing studies have grouped NLVs into two, possibly three, genogroups and 15 genotypes, while SLVs are grouped into 4 genotypes (Vinje and Koopmans 2000 Koopmans *et al.*, 2001). The use of molecular genotyping studies has begun to elucidate transmission mechanisms and geographic and temporal trends in occurrence of disease, but many questions regarding differences in virulence, induction of immune responses and cross-protection remain to be investigated.

Astroviruses

Astroviruses were first described in 1975 as a result of EM studies of an outbreak of diarrhoea in a maternity unit and a survey into the etiology of diarrhoea in childhood in a Scottish city (Madeley and Cosgrove 1975). Subsequently astroviruses were experimentally transmitted to adult volunteers and adapted to growth in foetal LLCMK2 cells. Restrictions on the availability and use of foetal tissue limited the cultivation of astroviruses and therefore studies were confined to a few laboratories that had access to electron microscopes. The concentration of excreted astroviruses appears to be lower than for other enteric viruses, so immune electron microscopy a relatively insensitive technique, identified these agents in 3% of cases of childhood diarrhoea. However, the use of more sensitive techniques has

identified these agents in 3–9% of childhood diarrhoea in several prospective studies (Glass *et al.*, 1996 Monroe *et al.*, 2001).

Clinically, these viruses cause similar symptoms to caliciviruses. Astroviruses have been isolated from birds, cats, dogs, pigs, sheep, cows and man. There are at least 7 human astrovirus serotypes (Monroe *et al.*, 2001). Like rotaviruses, astrovirus infections occur through the year with peaks in the winter months. Infections have been shown to occur mainly in childhood. A serological survey of 87 with children for antibody prevalence to astrovirus in England found that 67% of the children became seropositive by the age of 4 years and 87% by age 10. Other studies showed that most of the cases of infection were detected in children under five years of age with the majority of the children being under 1 year of age. Outbreaks of astrovirus infection involving children and elderly patients have been described and prolonged excretion documented in immunosuppressed, immunodeficient and AIDS patients. Significantly higher seroprevalence rates of astrovirus have been reported in adults exposed to contaminated water compared with a control group (Cook and Myint 1995). Studies have shown that astrovirus type 1 is the prevalent strain in the UK accounting for 65% of the cases. However this situation may be changing with the emergence of more cases of astrovirus type 4. In contrast, type 2 was the most prevalent in Mexico (31%) and type 1 relatively rare (6%) and there are reports of outbreaks of foodborne astrovirus infection in Japan involving thousands of children and adults associated with astrovirus type 6.

Adenoviruses

Adenoviruses are widely recognized causes of respiratory, ocular, and genitourinary infections. However, serotypes 40 and 41 (previously called fastidious enteric adenoviruses) primarily affect the gut, contributing to 5%–20% of hospitalizations for childhood diarrhea in developed countries (Uhnou *et al.*, 1984). Enteric adenoviruses have also been identified in paediatric gastroenteritis in developing countries (Bern and Glass 1994; Wadell *et al.*, 1987). Serotypes predominantly associated with human infections include h 40, h 41, which belong to subgenus F and occasionally h 31 in subgenus A. Peak incidence is among children less than 2 years of age, but older children and adults may be infected, with or without symptoms. Infections occur throughout the year with no clear peaks (Kotloff *et al.*, 1989). Incubation is between 3 and 10 days, with illness lasting greater than or equal to 1 week, longer than for other enteric viral pathogens (Uhnou *et al.*, 1984, Wood *et al.*, 1988). Diarrhoea is more prominent than vomiting or fever, and respiratory symptoms are often present. Person-to-person transmission is presumably the principal

mechanism for the spread of infection. Asymptomatic shedding has been documented, but generally infectivity parallels symptomatic disease (Kotloff *et al.*, 1989). Food and water have not been reported as vehicles. Reported outbreaks have tended to occur in hospitals or day-care settings, and all have involved children. Adult contacts were infrequently affected (Chiba *et al.*, 1990).

Pestivirus

A study on an Arizona Indian reservation showed that 23% of specimens from children less than 2 years of age with gastroenteritis of unknown etiology were antigen-positive for pestivirus, compared with 3% of controls. Illness was relatively mild, and duration was 3 days (Yolken *et al.*, 1989). Antibody studies of serum samples from Arizona, Maryland, and Peru suggested that 30%–50% of children and adults had been infected, with peak exposure occurring at less than 2 years of age (Yolken *et al.*, 1988).

Picobirnavirus

Reports from Brazil documented human cases of diarrhea caused by picobirnavirus, which had been thought to be a cause of diarrhoea only in animals (Pereira *et al.*, 1988). The importance of this pathogen is unknown, but it has been found in association with HIV and *Cryptosporidium* infected individuals (Gallimore *et al.*, 1995).

Parvovirus

Parvovirus-like particles have been identified by electron microscopy in stool specimens of both well and ill persons in Britain (Flewett *et al.*, 1974). The relationship of these particles to disease is unclear, but they have been associated with shellfish-related outbreaks of gastroenteritis (Appleton 1987).

Enteroviruses

Enteroviruses cause a wide spectrum of disease, in which gastroenteritis plays a minor role (Birch *et al.*, 1977). Although the entry of polio, coxsackie,¹ echo, or other enteroviruses through the gut may cause incidental mild diarrheal symptoms, the spread of the virus through the bloodstream to other organs (e.g., central nervous system, heart, pleura, pancreatic islets) produces major disease manifestations. Although reports have linked some enteroviruses to illnesses in which diarrhea was the sole symptom, an outbreak or case of gastroenteritis should not be attributed to an enterovirus merely because it was isolated in the stool of an affected person.

Toroviruses

Toroviruses are known causes of diarrhoea among cattle, and identification in human specimens has been reported (Beards *et al.*, 1987). In particular, toroviruses have been identified in 35% of paediatric patients with gastroenteritis and only 14.5% of controls, and patients with toroviruses were more likely to be immunocompromised than those with other viruses (Jamieson *et al.*, 1998).

Coronaviruses

Coronaviruses are well-established causes of diarrhoea in animals and respiratory disease in humans. These viruses have been identified in the stool of persons with gastroenteritis (usually children less than 2 years of age), but human controls have been found to shed them with higher frequency (Kidd *et al.*, 1989; Mathan *et al.*, 1975), raising doubt about their etiologic role in human diarrhoea. Coronaviruses have been detected most frequently in the southwest; one group reported that more than two-thirds of diarrheal stools examined by electron microscopy over an 8 years period contained such viruses, although no comparison was made with specimens from well persons (Payne *et al.*, 1986). Worldwide, coronaviruses have been detected at highest rates in situations of poor sanitation.

CHARACTERISTICS OF VIRUSES CASING ACUTE GASTROENTERITIS

Viruses that cause gastroenteritis include those which have double-stranded RNA, single stranded RNA and DNA genomes (Table 2), and appear to have evolved distinct from each other. Comparison of these viruses indicate genomic and antigenic diversity that plays an important role in determining the epidemiologic pattern of disease. Viruses with limited numbers of distinct serotypes cause infections that result in a good protective immune response in the host. In contrast, viruses with marked antigenic diversity such as the human caliciviruses infect humans throughout their lives.

Although all viruses causing gastroenteritis have family members that infect other species, animal reservoirs for human strains and human disease are not seen with these agents. There is some evidence from molecular studies that strains currently circulating in humans may have arisen due to genetic mixing with animal derived strains (Dastjerdi *et al.*, 1999; Nakagomi and Nakagomi 1989).

Caliciviral structure and genome organization

In 1972, Kapikian *et al.* used immune electron microscopy (IEM) to identify 27 nm viral particles, the Norwalk agent, in a fecal filtrate used to induce

Table 2 Characteristics of the viruses causing acute gastroenteritis

<i>Virus</i>	<i>Family</i>	<i>Size (nm)</i>	<i>EM shape</i>	<i>Nucleic acid</i>	<i>Genome organisation</i>	<i>Characterization</i>
Rotavirus	<i>Reoviridae</i>	70	Wheel-shaped (rota),	dsRNA	11 segments	Groups A, B, C; Group A has 2 subgroups (I, II); multiple serotypes, classified on the basis of two outer capsid proteins (P and G) Two main genogroups: 'Norwalk-like viruses' and 'Sapporo-like viruses', each with multiple distinct antigenic clusters 8 serotypes
Calicivirus	<i>Caliciviridae</i>	28-35	Small round structured	ss(+)-RNA	3 ORFs	
Astrovirus	<i>Astroviridae</i>	28-33	SRSV star-shaped morphology	ss(+)-RNA	2 ORFs	
Adenovirus	<i>Adenoviridae</i>	80	Icosahedral	dsDNA		Enteric types 40,41, 31 and types 42-48
<i>Other viruses</i>						
Torovirus	<i>Toroviridae</i>		Torus-shaped pleiomorphic	ss(+)-RNA		Role in human disease unknown
Picobirnavirus?			Small round virus	dsRNA	2 segments	Role in human disease unknown
Enterovirus 22?			Small round virus	ss(+)-RNA		Genetically distinct from Other enteroviruses
Aichi virus	<i>Picornaviridae</i>		Small round virus	ss(+)-RNA		Cause of outbreaks in Japan

ORF, open reading frame.

illness in human volunteers (Kapikian *et al.*, 1972). Later studies demonstrated that other small, round-structured viruses (SRSVs) morphologically similar to NV were associated with outbreaks of gastroenteritis. Attempts to propagate these agents in cell culture and organ culture were unsuccessful. In 1990, Jiang *et al.* provided molecular evidence that NV is a calicivirus by demonstrating that the viral genome consists of positive-sense, single-stranded, polyadenylated RNA (Jiang *et al.*, 1990). Subsequent elucidation of the complete sequence of NV and related SRSVs confirmed the genetic relatedness of these viruses to other caliciviruses.

The virions are composed of a single major capsid protein. The structure of baculovirus-expressed Virus Like Particles (VLPs) has been resolved by electron cryomicroscopy and computer image processing as well as by X-ray crystallographic methods (Prasad *et al.*, 1999). The capsid exhibits a $T = 3$ icosahedral symmetry. The major structural protein folds into 90 dimers that form a shell domain from which arch-like capsomers protrude. A key characteristic of this architecture is 32 cup-shaped depressions at each of the icosahedral fivefold and threefold axes. These cup-like depressions are more prominent in some strains (particularly the SLVs), leading to the characteristic “Star of David” appearance from which caliciviruses get their name. The name calicivirus is derived from the Latin calyx, meaning cup or goblet, and refers to the cup-shaped depressions visible by electron microscopy. Recently, the International Committee on the Taxonomy of Viruses established and approved four genera within the family Caliciviridae, including two human calicivirus (HuCV) genera (Green *et al.*, 2000). These nonenveloped viruses have a diameter of 27 to 40 nm by negative-stain electron microscopy and a buoyant density of 1.33 to 1.41 g/cm³, and they contain a positive-sense polyadenylated single-stranded RNA of approximately 7.6 kb. The two HuCV genera currently have the tentative names of “Norwalk-like viruses” (NLVs; type strain, NV [Hu/NLV/Norwalk virus/8FIIa/1968/US]) and the “Sapporo-like viruses” (SLVs; type strain Sapporo virus [Hu/SLV/Sapporo virus/1982/JA]). The proposed nomenclature for HuCVs is species infected/virus genus/virus name/strain designation/year of isolation/country of isolation.

The genome of NLVs is organized in three major open reading frames (ORFs). Figure 1 compares the genomes of three positive sense RNA viruses, caliciviruses, astroviruses and enteroviruses. For NV, the first ORF at the 5' end encodes a large polyprotein of 1738 amino acids (aa) with a predicted molecular weight of 193.5 (193.5 K). This polyprotein contains short motifs of similarity with the 2C (helicase), 3C (cysteine protease), and 3D (RNA-dependent RNA polymerase) proteins of picornaviruses. Thus, the 5' end of the genome of the NLVs codes for a precursor of the nonstructural proteins. ORF2 encodes a 530-aa (56.6 K) protein, the capsid

protein. The ORF2 protein expressed in insect cells self-assembles into VLPs. ORF3 at the 3' end of the genome is predicted to code for a small protein of 212 aa (22.5 K) with a very basic charge (isoelectric point of 10.99). The ORF3 protein does not have sequence similarity with any other proteins in the GenBank database, and its function remains unknown. Sequence information has been used to identify relationships between strains of NLVs. Most comparisons have focused on the region of the genome encoding the viral RNA-dependent RNA polymerase or on the capsid protein gene, but comparisons of other genomic regions have also been made. Such comparisons have allowed the further subdivision of viruses in the NLV genus into two genogroups and viruses in both HuCV genera into clusters (Green *et al.*, 2000). Based on RdRp and Capsid gene NLVs are classified into 3 genogroups: of which Genogroup I consist of Norwalk virus (NV), Desert Shield virus (DSV), Southampton virus (SOV) and consists of 5 clusters; Genogroup II consist 10 clusters in which the majority of viruses are Hawaii virus (HV), Mexico virus (MV), Snow Mountain virus (SMV), Genogroup III consist of Jena virus (JV), of these genogroup I&II infects only to humans but JV infects calf. Based on RdRp region SLVs are classified into 3 genogroups: Sapporo virus (SV), London-92 virus (LV) and Parkville virus (PV) (Green *et al.*, 2000). Norwalk-like viruses are classified into 9 antigenic types: UK1-1, UK1-2, UK1-3, UK2-12, UK3-15, UK3-17, UK 4-20 and UK4-22 based on SPIEM.

Taunton agent (TV) is the prototype of UK1, Norwalk agent (NV) of UK2, Hawaii agent (HWA) of UK3 and Snow Mountain agent (SMA) of UK4

Astrovirus structure and genome organization

Human astrovirus is the prototype of the Astroviridae, a family of non-enveloped positive sense RNA viruses. By direct electron microscopy, astroviruses recovered from stool display a distinctive surface star-like appearance. However, in viruses propagated in cell culture, alkaline treatment is required for the characteristic structure. Recent structural analysis using cryoelectron microscopy shows spherical particles of uniform size with clearly visible surface spikes. Three dimensional reconstruction from these images shows a smoothly rippled, solid capsid shell with a diameter of 330 Å and 30 dimeric spikes centred at the twofold axis of symmetry, that extend about 50 Å from the surface. Inside the capsid, the genomic RNA appears to assume a partial icosahedral configuration (Matsui *et al.*, 2001).

The genome of astrovirus consists of plus-sense, single stranded RNA, 6.8 kb in length, excluding the poly(A) tract at the 3' end, organized in 3 ORFs. ORFs 1a and 1b at the 5' end encode motifs for non-structural proteins, including a viral protease and a nuclear localization signal in ORF

1a and an RNA dependent RNA polymerase in ORF 1B (Jiang *et al.*, 1993; Lewis *et al.*, 1994). ORF2 encodes an 87 kDa structural protein that is the precursor of the mature capsid proteins (Bass and Qiu, 2000). All serotypes have at least three capsid proteins, P1, P2 and P3, with the P2 protein carrying the group-reactive epitopes and the P3 protein specifying serotype (Belliot *et al.*, 1997).

Adenovirus structure and genome organization

All adenovirus particles are non-enveloped, 60–90 nm diameter, with icosahedral symmetry easily visible in the electron microscope by negative staining and are composed of 252 capsomers: 240 hexons and 12 pentons at the vertices of an icosahedron (2-3-5 symmetry). Individual protomers can be isolated by progressive chemical disruption of purified virus particles. The hexons consist of a trimer of polypeptide II with a central pore; VI, VIII and IX are minor polypeptides also associated with the hexon, thought to be involved in stabilization and/or assembly of the particle. The pentons are more complex; the base consists of a pentamer of peptide III, 5 molecules of IIIa are also associated with the penton base. The pentons have a toxin-like activity, purified pentons causing cytopathic effect in the absence of any other virus components. A trimeric fibre protein extends from each of the 12 vertices (attached to the penton base proteins) and is responsible for recognition and binding to the cellular receptor. A globular domain at the end of the adenovirus fiber is responsible for recognition of the cellular receptor.

The core of the particle contains at least 4 proteins, TP (Terminal Protein) covalently attached to the 5' ends of the genome strands, V (180 copies/particle) and VII (1070 copies/particle) are basic proteins non-covalently associated with the genome forming a “chromatin-like” substance. The genome is linear, non-segmented, double-stranded DNA, 30–38 kbp which has the theoretical capacity to encode 30–40 genes. Genome structure as determined by cross-hybridization and restriction mapping is one of the characters used to assign viruses to groups (70–95% homology within groups, 5–20% homology between groups). The terminal sequences of each strand are inverted repeats, hence the denatured single strands can form “panhandle” structures (100–140 bp). There is a 55 kD protein covalently attached to the 5' end of each strand.

Coronavirus Structure and Genome Organization

Coronavirus virions are spherical to pleomorphic enveloped particles. The envelope is studded with projecting glycoproteins, and surrounds a core consisting of matrix protein enclosed within which is a single strand of positive-sense RNA ($M_r 6 \times 10^6$) associated with nucleoprotein. The envelope glycoproteins are responsible for attachment to the host cell and

also carry the main antigenic epitopes, particularly the epitopes recognized by neutralizing antibodies. OC43 also possesses a haemagglutinin. The coronaviruses were originally grouped into the family *Coronaviridae* on the basis of the crown or halo-like appearance given by the glycoprotein-studded envelope on electron microscopy. This classification has since been confirmed by unique features of the chemistry and replication of these viruses. Most human coronaviruses fall into one of two groups: 229E-like and OC43-like. These differ in both antigenic determinants and culturing requirements: 229E-like coronaviruses can usually be isolated in human embryonic fibroblast cultures; OC43-like viruses can be isolated, or adapted to growth, in suckling mouse brain. There is little antigenic cross-reaction between these two types.

Coronaviruses have a helical nucleocapsid that consists of 27–32 kb plus strand RNA genome encapsidated by the nucleocapsid phosphoprotein N. It is thought that human coronaviruses enter cells, predominantly, by specific receptors. Aminopeptidase-N and a sialic acid-containing receptor have been identified to act in such a role for 229E and OC43 respectively. After the virus enters the host cell and uncoats, the genome is transcribed and then translated. A unique feature of replication is that all the mRNAs form a “nested set” with common 3' ends; only the unique portions of the 5' ends are translated. There are 7 mRNAs produced. The shortest mRNA codes for the nucleoprotein, and the others each direct the synthesis of a further segment of the genome. The proteins are assembled at the cell membrane and genomic RNA is incorporated as the mature particle forms by budding from internal cell membranes (Holmes, 2001).

Torovirus structure and genome organization

Toroviruses belong to the family *Coronaviridae*, order *Nidovirales*. The virions are enveloped, slightly pleomorphic, spherical, or kidney-shaped, 120–140 nm in diameter; up to 170 nm long. The surface projections of the envelope are distinct; club-shaped (10–20 nm long), spaced widely apart and dispersed evenly over all the surface. The nucleocapsids are rod-shaped, with no clear modal length, 104 nm long, 9 nm in diameter, or 11–13 nm in diameter. The symmetry is helical or tubular with a molecular mass (M_r) of virion is 400×10^6 . Under *in vitro* conditions, the virions of some viruses are stable in acid environment (pH 3). The virions are sensitive to heat, lipid solvents, non-ionic detergents, formaldehyde, and oxidizing agents. The genome consists of one molecule of linear positive-sense single stranded RNA. The total genome length is 20000 nt. The 5' end of the genome has a cap, with a poly (A) tract at the 3' end. Sub-genomic mRNA found in infected cells. There are three structural virion proteins. The surface glycoprotein (or spike, S, 20 kDa) is responsible for attachment to cells, hemagglutination and membrane fusion. It has a carboxy-terminal

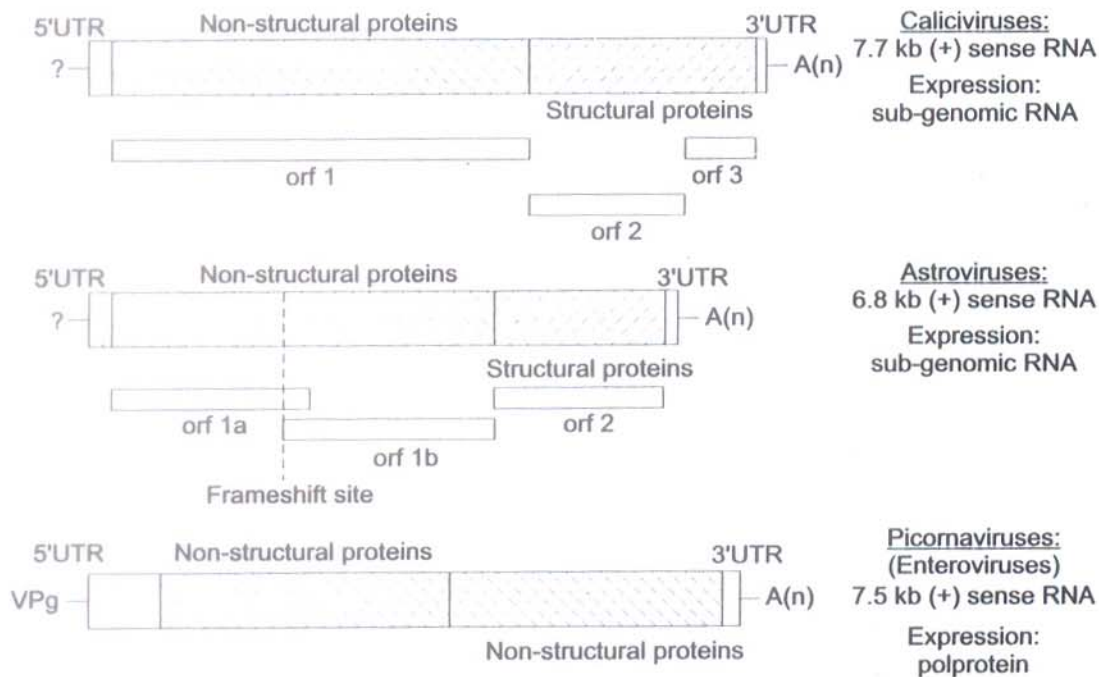


Fig. 1 Comparison of genomic organisation of three positive sense RNA viruses implicated in diarrhoeal disease.

half with a coiled-coil structure. The integral membrane protein (M, 27 kDa) spans the virus envelope three times with only 10% protruding at the virion surface. The nucleocapsid protein is N and has a MW of 19 kDa. The virion structural proteins are glycosylated. The virus has distinct antigenic determinants on envelope and spikes, that correspond to each of the major structural glycoproteins S, M, and N. Antigenic specificity of virion can be determined by neutralization tests (S), or complement fixation tests (M). Protective immunity is induced in the form of complement independent neutralizing antibodies (Holmes 2001).

DIAGNOSTIC ASSAYS

During the 1970s and 1980s, tests for the diagnosis of non-cultivable viral infections were designed using reagents from previously infected humans. The inability to propagate NV and related viruses in cell culture also prevented the production of animal hyperimmune sera. Thus, stools of acutely infected individuals served as a source of virus antigen, and convalescent-phase sera from infected individuals were used as hyperimmune sera. These restrictions limited the general availability of these diagnostic tools to only a few research laboratories. However, a number of tests were developed with these reagents and used to begin to define the epidemiology of human viral gastroenteritis. These included direct electron microscopy (requires virus concentrations of at least 10^6 per ml of stool), immune electron microscopy or IEM, solid-phase IEM,

immune adherence hemagglutination assay, radioimmunoassay, enzyme immunoassay and Western blot assay (Atmar and Estes 2001). The development of sensitive diagnostic assays based on molecular methods in the 1990s have advanced our understanding of the epidemiology and clinical importance of gastroenteritis viruses, particularly the caliciviruses. The lack of reliable assays for detection of novel agents including Group C rotaviruses, toroviruses, picobirnaviruses and the Aichi agent in human samples, have held back our understanding of the epidemiology and causal role of these agents.

Antigen Detection

Commercial antigen-detection kits for enteric adenoviruses and astroviruses are widely available, inexpensive, and permit rapid viral diagnosis. Only small amounts of stool are required for the tests, and samples may be frozen before testing. Kits vary widely in range of sensitivities (70%–100%) and specificities (50%–100%) (Dennehy *et al.*, 1988; Thomas *et al.*, 1988). Newborns and breast-feeding children have particularly high false-positive rates. Confirmatory testing should be performed in any case in which disease would be unusual as well as periodically to validate the reliability of the assay employed. Since adenoviral diarrhoea affects mainly children less than 2 years of age and because outbreaks involving adults have never been reported, the diagnostic value outside the preschool-age group is also limited.

Although commercial assays are not yet available for caliciviruses, the successful cloning of NV led to the development of new reagents and methods for the diagnosis of infections caused by HuCVs. When the NV capsid protein was expressed in a baculovirus expression system, VLPs were generated. These VLPs were subsequently shown to be morphologically and antigenically similar to native virus particles (Jiang *et al.*, 1992). The VLPs were used to immunize different animal species to produce polyclonal and monoclonal immune sera that could then be used to establish EIA-based diagnostic assays. Virus sequence was used to design primer pairs for the detection of HuCVs using reverse transcription (RT)-PCR (Jiang *et al.*, 1993).

EIA with hyperimmune animal sera: The production of NV VLPs provided sufficient quantities of viral capsid antigen to allow the generation of hyperimmune sera in mice, guinea pigs, and rabbits. Hyperimmune sera from these animals have NV-specific antibody titers of 1:256,000 to >1:1,000,000. Subsequently, VLPs have been produced for other HuCVs, including Mexico virus (MX), SMA, HV, Desert Shield virus, Toronto virus (TV), Grimsby virus (GRV), Sapporo virus, Southampton virus, and Lordsdale virus (LV). Polyclonal hyperimmune animal sera produced by

immunization of different animal species with VLPs have been used to develop antigen detection EIAs for use in clinical specimens. These immune sera have been quite specific, detecting homologous recombinant VLPs in an EIA format but not reacting with heterologous VLPs (Atmar and Estes 2001).

A limitation of these antigen detection assays was recognized when the assays were applied to clinical samples containing other HuCVs. For example, the antigen detection assay that utilizes hyperimmune sera raised to rNV VLPs only detects a subset of genogroup I NLVs and does not detect genogroup II NLVs. Only the most closely related viruses in genogroup I (90% aa identity in the polymerase region) were detected in this assay. Similarly, the antigen detection assay that utilizes hyperimmune sera raised to rMX VLPs is most efficient at detecting genogroup II NLVs that are the most closely related to MX and does not detect genogroup I viruses and the genogroup II NLV GRV in stool samples, and conversely, an assay using hyperimmune serum raised against rGRV VLPs does not detect MX in stool. These assays also do not detect SLVs (Hale *et al.*, 1996, Hale *et al.*, 1999). Thus, the lack of an EIA that is broadly reactive with a range of HuCVs has limited the utility of these assays.

EIA with MAbs: Monoclonal antibodies (MAbs) have been prepared using native NV, native SMA, and rNV VLPs. Similar to what was seen with polyclonal sera, these MAbs are often type specific, recognizing the capsid protein of the immunizing virus but not that of other NLVs. The MAbs have been evaluated in limited studies for the detection of virus in stool samples. The description of a common epitope for genogroup I viruses leaves open the possibility that a similar common epitope may be present in genogroup II viruses. If all NLVs contain one or a limited number of common epitopes, the development of a broadly reactive antigen detection EIA will be possible. Such assays are desirable because large numbers of samples could be tested in a rapid and cost-effective manner.

Antibody Detection

Persons infected with a viral agent of gastroenteritis will usually have a rise in antibodies to that virus. For the Norwalk agent, preexisting IgG antibodies to the virus in the majority of populations studied render a single specimen insufficient to document infection. If at least half of affected persons in an outbreak have a fourfold rise in specific antibody titers, the Norwalk agent can be designated as etiologic. Titers may begin to rise by the fifth day after onset of symptoms, peak at approximately the third week, and often begin to fall by the sixth week. Hence, the acute-phase serum should be drawn within the first week and the convalescent-phase serum during the third to sixth weeks. Diagnostic assays for IgM and IgA

antibodies to Norwalk virus have been used on an experimental basis (Erdman *et al.*, 1989a, b). One disadvantage of serologic diagnosis is that patients are often reluctant to have serum drawn a month after a brief, self-limited illness. Furthermore, because this class of viruses cannot be cultivated, the supply of antigen for antibody testing is limited to a few research laboratories and cannot be offered for routine screening. In addition, antibodies can be detected to the Norwalk virus only, not the full spectrum of Norwalk-like agents that may cause disease

Electron Microscopy

Under an electron microscope, a virus can be identified by its characteristic morphology in a stool specimen. The technique is highly specific but requires substantial resources. Since an electron microscope scans a field of approximately 1 millionth of a millilitre there must be at least 1 million viruses/millilitre of stool for a detection to be made. Such levels of excretion are normally present only during the first 48 hours of viral diarrhea. With immune electron microscopy (IEM), the sensitivity of normal transmission electron microscopy can be improved 10–100 times. In one technique, the grid to be examined is coated with convalescent-phase serum before the stool specimen is applied; a high titer of virus-specific antibody tends to hold aggregates of homologous virus in the field, thereby enhancing diagnostic yield. Because reagents are scarce, this technique for diagnosing viral gastroenteritis is limited to a few centers globally.

Culture

Enteric adenoviruses, and astrovirus can be cultured in research centers, but the techniques are not well suited for routine diagnosis. The other known major viral enteric pathogens cannot yet be cultivated. Enteroviruses can be cultivated but are not thought to be important causes of diarrhea.

Hybridization probes

Hybridization assays have been developed for adenoviruses, but they are less sensitive than antigen-detection techniques (Hammond *et al.*, 1987). Hybridization assays can also be used for the confirmation of nucleic acid amplification by hybridizing labelled probes to the amplified DNA. The most common include dot or slot blot hybridization, liquid hybridization, and Southern blot hybridization (Atmar and Estes 2001). In these assays, a virus-specific probe is labeled and hybridized with the PCR products, and the presence or absence of the label is detected. Some of the more common labels include ^{32}P , digoxigenin, and biotin. One of the limitations of

hybridization assays when applied to NLV RT-PCR assays is that the variability of the genomic sequence in the NLVs makes it difficult to select a single or even a small number of probes that can detect all possible NLV sequences.

Nucleic Acid Detection

Nucleic acid detection assays have been developed in the last decade since the cloning of the genome of caliciviruses, adenoviruses and astroviruses. For caliciviruses, knowledge of the sequence of the NV genome led to the design of primers from the polymerase region that were able to amplify fragments of other NLVs and SLVs, and this led to sequencing of the complete genomes of many HuCVs. Although there have been a few reports of the use of hybridization assays, the primary nucleic acid detection assay that is used is RT-PCR. RT-PCR is currently being used worldwide because of the lack of a commercially available, broadly reactive EIA. Only a few hybridization assays have been described for the detection of HuCVs. This is most likely due to the availability of the more sensitive RT-PCR assays at the time that cDNAs for HuCVs first became available. Clinical samples frequently contain substances that can inhibit the enzymatic activity of the reverse transcriptase and DNA polymerase enzymes used in RT-PCR assays. Thus, it is usually necessary to partially purify viral nucleic acids or otherwise prepare the sample prior to the performance of the RT-PCR assay. Two major considerations in selecting an extraction method are its efficiency of viral nucleic acid recovery and its ability to remove or inactivate RT-PCR inhibitors. Secondary considerations include the ease of performance of the method and the number of samples that can be processed at one time. The sensitivity and specificity of RT-PCR assays depend in large part on primer selection. Several factors affect the ability of a primer pair to detect a given strain, including primer sequence, the amount of virus present in the sample to be assayed, and the temperature used for primer annealing during the PCR amplification process. In particular for caliciviruses, the genetic diversity of NLVs and SLVs has made it difficult to select a single primer set with adequate sensitivity and specificity to detect all NLVs. In general, regions of the genome with the greatest degree of conservation between strains within the genera and within genogroups have been targeted for amplification and primer design. But even within these regions, the nucleotide identity can be as little as 36% (2C helicase region) to 53% (3D polymerase region) between strains of different genogroups. Within a genogroup of NLVs, greater conservation is seen, but the nucleotide identity between strains can still be as little as 60 to 64%. These observations have led to the design and use of primer sets targeting multiple areas of the viral genome.

The majority of primers have been designed to amplify the most conserved region of the genome, the RNA-dependent RNA polymerase region. Although a number of primer pairs have been described, those described by Ando *et al.* (1995), Green *et al.* (1995), and Le Guyader *et al.* (1996) have been the most frequently used. Occasionally, primers based on the sequence of a locally circulating strain have performed better than other primer pairs. Other regions of the viral genome have been targets of amplification, including the 2C helicase, the capsid region, and ORF3. The most common reason to target another area of the genome is to generate additional sequence data that might be useful in distinguishing or identifying unique viral strains. Sequence data from two regions of the viral genome have suggested that some caliciviruses may have evolved by undergoing recombination. Such events may make it necessary to analyze more than one region of the genome to characterize the relatedness of circulating viral strains in epidemiological studies.

PREVENTION AND CONTROL

Although essential in outbreak management, improved environmental hygiene (i.e., food, water, and sanitation) may be ineffective in endemic control of some of the known viral agents of gastroenteritis, perhaps because person-to-person transmission is the principal mechanism for the spread of infection. As a result, the population-based attack rate for these agents is thought to be the same (100%) in developed and developing countries, although disease caused by known agents tends to be acquired earlier in developing countries. The risk of death is highest in areas where medical care is least available and malnutrition is most prevalent.

Although person-to-person transmission is an important aspect of endemic disease, the initiating event for most outbreaks of viral gastroenteritis is contamination of a common source. In contrast to bacterial pathogens, enteric viruses cannot multiply outside their host; hence, the original inoculum into the common source determines infectivity. Among foods, the role of viral contamination has been best studied in shellfish. Shellfish that grow in fecally contaminated water concentrate enteric viruses in their tissues, and even harvests meeting bacteriologic standards of hygiene may contain viral agents. In addition, depuration (a technique in which shellfish are flushed with clean water treated with ultraviolet light) is less effective in viral than in bacterial decontamination. When foods other than shellfish are implicated in viral gastroenteritis outbreaks, the contamination has usually taken place near the point of consumption. An ill food handler was identified in nine of the 15 documented Norwalk outbreaks reported to CDC from 1985 to 1988 for which adequate epidemiologic data were available (CDC, unpublished

data). Foods that require handling and no subsequent cooking (e.g., salads) constitute the greatest risk. The long list of foods implicated in outbreaks of viral gastroenteritis reflects the variety of foods that are handled by food-service personnel and the low infectious dose (10–100 particles) of most viral agents of gastroenteritis, rather than peculiar viral tropisms. In contrast to the factors important in amplifying bacterial contamination, practices such as leaving foods unrefrigerated or warming them for prolonged periods are not direct risk factors for increased viral transmission because the viruses do not multiply outside the human host.

Outbreaks of viral gastroenteritis have been associated with various sources of contaminated water, including municipal water, well water, stream water, commercial ice, lake water, and pool water. Studies have documented that the Norwalk agent can remain highly infective despite 30 minute exposure to concentrations of chlorine as high as 6.25 mg/L, and viral RNA can be detected and amplified even after 60 minutes of exposure to 5000 ppm of sodium hypochlorite (Barker 2001). This resistance may explain why the Norwalk agents are prominent in outbreaks of waterborne disease.

Vaccines

Vaccines against viral gastroenteritis continue to be an important area for research. Trial studies on Norwalk virus have shown that virus-like particles expressed in potatoes can induce an immune response in animal models and in humans (Estes *et al.*, 2000).

SUMMARY

The application of newer diagnostic tests has changed our understanding of the epidemiology of infections caused by enteric viruses, as agents of both endemic and epidemic disease. The use of molecular tests has established the increasing important role of astroviruses and caliciviruses. NLVs are now recognized to be the principal cause of outbreaks of nonbacterial gastroenteritis, and new estimates suggest that they are the most common cause of foodborne illnesses. In addition, the duration of virus shedding has been found to be longer than previously recognized, providing a potential explanation for the occurrence of outbreaks traced to postsymptomatic individuals.

As has been mentioned earlier, India has little recent molecular data on the role of viruses in gastroenteritis (Kang *et al.*, 2000, Girish *et al.*, 2002). In addition, with the international recognition of the role of Norwalk-like viruses as the major cause of food related illness, it is imperative that we obtain data on the burden of disease, transmission and efficacy of methods of prevention of this major group of pathogens. The role of other enteric viruses in the causation of gastroenteritis and the continued awareness that

not all causes of enteric infection have been identified must be emphasized in future studies.

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